REMARKS

The present application relates to inbred maize line PH8CW. Claims 1-34 are pending in the present application. Claims 23-26 have been amended. No new matter has been added by way of amendment. Applicants respectfully request consideration of the claims in view of the following remarks.

Detailed Action

Applicants acknowledge that because this application is eligible for continued examination under 37 C.F.R. § 1.114 and the fee set forth in 37 C.F.R. § 1.17(e) has been timely paid, the finality of the previous Office Action has been withdrawn pursuant to 37 C.F.R. § 1.114. Applicants further acknowledge that Applicants' submission filed on April 11, 2006 has been entered.

Applicants further acknowledge that the terminal disclaimer filed on January 5, 2006 has been reviewed and accepted.

Rejections Under 35 U.S.C. § 112, Second Paragraph

Claims 11-12, 23, 25-28, and 32-34 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regards as the invention. The Examiner states claim 11 is indefinite "for omitting essential steps." *See* Office Action, pp. 2-3.

Applicants traverse this rejection. Applicants have included "repeating steps (c) and (d) to produce backcross progeny plants that comprise the desired trait and comprise at least 95% of the alleles of inbred line PH8CW at the SSR loci listed in Table 4" in claim 11. Applicants further assert the use of molecular marker profiles by those of ordinary skill in the art in backcrossing is also clearly supported by the scientific literature. For example, see Ragot, M. *et al.* (1995) Marker-assisted backcrossing: a practical example, in *Techniques et Utilisations des Marqueurs Moleculaires (Les Colloques*, Vol. 72, pp. 45-56 (attached as Appendix 1), and Openshaw *et al.*, (1994) Marker-assisted Selection in Backcross Breeding, Analysis of Molecular Marker Data, pp. 41-43 (attached as Appendix 2). Specifically, Ragot *et al.* states in the first sentence of the summary "[t]hat molecular markers allow fast recovery of recurrent parent genotype in backcross programs is undisputed," and, in the first sentence of the introduction,

"[b]ackcrossing has been a common breeding practice for as long as elite germplasm has been available." Therefore, Applicants have claimed in the manner used by those of ordinary skill in the art to characterize backcross conversions.

Claim 12 is rejected as rejected as indefinite as depending from rejected claim 11.

Applicants traverse this rejection for the reasons asserted *supra.* Claim 12 is definite and does include the essential method steps of claim 11.

Regarding claims 23-28, the Examiner states that the claims "do not incorporate all elements of the parent claim (13)," specifically that the "plant of parent claim 13, PH8CW, does not contain a single locus conversion or any of the locus conferring traits as listed in claims 26-28." *See* Office Action, p. 3.

Applicants respectfully traverse. Claim 13 specifically claims a maize plant having all the physiological and morphological characteristics of inbred line PH8CW. Claim 13 encompasses maize plants having the characteristics of inbred line PH8CW. Claims 23-28 claim the maize plant of claim 13 with these <u>additional</u> limitations, which are not necessarily present in the maize plant of claim 13. The presence of these additional limitations does not mean that claims 23-28 do not possess all limitations of claim 13; these claims still require a maize plant having the physiological and morphological characteristics of inbred line PH8CW. Because claims 23-28 do incorporate all elements of claim 13, they are in accordance with the requirements of § 112, second paragraph.

The Examiner further states that claims 26-27 are indefinite in the "recitation of a gene conferring 'male sterility' ... however, the parent is male fertile." *See* Office Action, p. 3.

Applicants respectfully traverse. It would be understood by one of ordinary skill in the art that the deposited line can be manipulated and made male sterile by methods such as backcrossing, as described in the specification. *See, e.g.*, specification, pp. 2-4. "It should be understood that the inbred can, through routine manipulation by detasseling, cytoplasmic genes, nuclear genes, or other factors, be produced in a male-sterile form." *See* specification, p. 30, ll. 26-28. One of skill in the art also understands that transgenes can be incorporated into the inbred line in a similar manner. *See* specification, pp. 34-40. Male sterile conversions have been made to inbred lines since the 1950's, and transgenic conversions have been made to inbred lines since the early 1990's. Both are routinely made, and the language and meaning of these claims are well understood by plant breeders. The primary purpose of the requirement of definiteness of

claim language is to "ensure that the scope of the claim is clear so the public is informed of the boundaries." MPEP § 2173. That objective has been satisfied by claims 26-27.

Claim 32 is rejected as indefinite in the recitation of "using" without any active method steps. *See* Office Action, p. 3.

Applicants traverse this rejection. The specification states "[p]lant breeding techniques known in the art and *used* in a maize plant breeding program include, but are not limited to, recurrent selection, backcrossing, pedigree breeding, restriction fragment length polymorphism enhanced selection, genetic marker enhanced selection, making double haploids, and transformation. Often a combination of these techniques are used." Specification, p. 3, ll. 25-30 (emphasis added). Therefore, Applicants assert that one of skill in the art would know the meaning of the term "using" in claim 32.

In light of the above amendments and remarks, Applicants respectfully request reconsideration and withdrawal of the rejections under 35 U.S.C. § 112, second paragraph.

<u>Rejections Under 35 U.S.C. § 112, First Paragraph</u>

Claims 1-12, 15-19, 21-26, and 29-34 stand rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. The Examiner asserts that the claims contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention. The Examiner states "Applicant has neither described a method of reproducing the disclosed F1 hybrid nor does Applicant indicate that the seed of said F1 hybrids of the inbred PH8CW have been deposited and are publicly available." *See* Office Action, p. 4-8.

Applicants respectfully traverse this rejection. Primers for the SSR markers listed in Table 4 are publicly available as stated in the present application. Applicants respectfully direct the Examiner's attention to p. 70, ll. 12-15 of the specification where it states that "[p]rimers used for the SSRs reported herein are publicly available and may be found in the Maize GDB using the World Wide Web prefix followed by maizegdb.org (maintained by the USDA Agricultural Research Service), in Sharopova *et al.* (Plant Mol. Biol. 48(5-6):463-481), Lee *et al.* (Plant Mol. Biol. 48(5-6); 453-461), or reported herein". A print out from the MaizeGDB web site using bnlg1014 as an example has been included with this response as Appendix 3. The print out

shows the extensive amount of marker information available on the MaizeGDB, which includes primer sequences and map information. As explained in the specification, primer sequences for the public SSR markers listed in Table 4 can be easily obtained through the world wide web.

Further, Applicants assert that at least 95% of the alleles of inbred line PH8CW disclosed in the SSR profile of Table 4 is an identifying physical characteristic that describes the genus of minor variants of inbred line PH8CW, including, but not limited to, single locus conversions produced through transformation or introgression. The SSR profile of PH8CW is disclosed for numerous markers distributed throughout the genome as indicated by the Bin number of the marker, which denotes the marker location. A plant comprising 95% of the alleles of PH8CW as disclosed in Table 4 would be produced, for example, by repeated backcrossing to PH8CW. A backcross conversion of PH8CW as claimed in the instant application is described as comprising 95% of the alleles disclosed in Table 4.

It is undisputed that fingerprinting with molecular markers is widely used for characterizing germplasm. Specifically, SSR profiles are known and can be practiced by one of ordinary skill in the art in maize breeding. One of ordinary skill has been enabled by the deposit to make and use minor variants of inbred corn line PH8CW, and one of ordinary skill in the art uses SSR markers to characterize backcross conversions of an inbred. Applicants have claimed in the manner used by those of ordinary skill in the art to characterize backcross conversions.

Applicants also point out that molecular marker methods are known to one ordinarily skilled in the art and the SSR profile of PH8CW can be obtained from the deposit, but notwithstanding, Applicants have also provided the SSR profile of PH8CW in the application. *See* specification, Table 4, pp. 71-74 and U.S. Patent No. 6,784,349; Table 4, column 43, l. 37 through column 45, l. 25, respectively. Applicants reiterate that according to *Enzo*, the deposit of a material in a public depository is an adequate description of that material for purposes of the written description requirement. *Enzo Biochem, Inc.*, 296 F.3d at 1325, 63 U.S.P.Q.2d at 1613. In addition, *Regents of University of California*, 119 F.3d at 1568, 43 U.S.P.Q.2d at 1406, teaches that claims may satisfy the written description requirement where they disclose "structural features commonly possessed by members of the genus that distinguish them from others." The Board of Patent Appeals & Interferences has also confirmed the sufficiency of a deposit for seed and plants in the case of *Ex Parte C,* 1992 WL 515817 p. * 5, 27 U.S.P.Q.2d 1492, 1496 (B.P.A.I. 1992), where it stated that "[t]he claimed soybean is described in the

specification to the extent that there is no question that appellant was in possession of the invention as of the time the instant application was filed. Because seed is to be deposited in a public depository, the specification is enabling and sets forth the best mode of carrying out the invention." Consistent with this principal, the Board of Patent Appeals & Interferences, in a case involving the written description requirement as applied to seed and plants, stated "[i]f in making the latter comment the examiner is requiring appellants to have reduced to practice each possible plant within the scope of the claims, such a position is legally incorrect. The specification need only teach one skilled in the art how to make and use the claimed invention. How the specification does so, whether by way of the written word or actual examples, is of no moment." *Ex parte Gerardu C.M. Bentvelsen et al.*, 2001 WL 1197757, p. *2 (B.P.A.I. 2001).

The Applicants further assert those of skill in the art utilize molecular markers, such as SSR's, to characterize plant genomes. As Applicants' clearly teach in the specification:

> To accomplish this goal, the maize breeder must select and develop superior inbred parental lines for producing hybrids. This requires identification and selection of genetically unique individuals that occur in a segregating population. The segregating population is the result of a combination of crossover events plus the independent assortment of specific combinations of alleles at many gene loci that results in specific genotypes. *See* specification, p. 8, ll. 14-19.

Further, Applicants teaches:

> In addition to phenotypic observations, the genotype of a plant can also be examined. A plant's genotype can be used to identify plants of the same variety or a related variety. For example, the genotype can be used to determine the pedigree of a plant. There are many laboratory-based techniques available for the analysis, comparison and characterization of plant genotype; among these are Isozyme Electrophoresis, Restriction Fragment Length Polymorphisms (RFLPs), Randomly Amplified Polymorphic DNAs (RAPDs), Arbitrarily Primed Polymerase Chain Reaction (AP-PCR), DNA Amplification Fingerprinting (DAF), Sequence Characterized Amplified Regions (SCARs), Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs) which are also referred to as Microsatellites, and Single Nucleotide Polymorphisms (SNPs). *See* specification, p. 20, ll. 11-20. .

Applicants also teach how the claimed backcross trait conversions are "routinely used and have a very high rate of success". *See* specification, p. 31, l. 18. Those plants and plant parts that are developed substantially benefiting from the use of inbred maize line PH8CW "comprising a single gene conversion, transgene, or genetic sterility factor, may be identified by having a

molecular marker profile with a high percent identity to PH8CW". *See* specification, p. 74, l. 13-15.

The use of molecular marker profiles by those of ordinary skill in the art in backcrossing is also clearly supported by the scientific literature. For example, see Ragot, M. *et al.* (1995) Marker-assisted backcrossing: a practical example, in *Techniques et Utilisations des Marqueurs Moleculaires (Les Colloques*, Vol. 72, pp. 45-56 (attached as Appendix 1), and Openshaw *et al.*, (1994) Marker-assisted Selection in Backcross Breeding, Analysis of Molecular Marker Data, pp. 41-43 (attached as Appendix 2). Specifically, Ragot *et al.* notes that "spectacular" progress toward the recurrent parent genotype was obtained with 61 RFLP markers. Ragot *et al.* also concludes that "recovery of the recurrent parent genotype could proceed even faster than in the experiment described herein, should the appropriate protocol and resources (population size, number and position of markers) be allocated." In the case at issue, over 125 markers have been provided. SSR markers have been demonstrated to be at least as reliable, if not more so, than RFLP markers. *See* J.S.C. Smith *et al.*, An Evaluation of the Utility of SSR Loci as Molecular Markers in Maize (Zea Mays L.): Comparisons with Data from RFLPS and Pedigree, Theor. App. Genet. 95:163-173 (1997) (attached as Appendix 4). Accordingly, it is clear that at least 95% identity based on over 125 SSR markers is more than sufficient to characterize the claimed backcross conversions of PH8CW to one of ordinary skill in the art.

Thus, SSR profiles are known and can be practiced by one of ordinary skill in the art. One of ordinary skill has been enabled by the deposit to make and use backcross conversions of inbred corn line PH8CW, and one of ordinary skill in the art uses molecular markers to characterize backcross conversions of an inbred line. Applicants have claimed in the manner used by those of ordinary skill in the art to characterize backcross conversions, and 95% identity based on over 125 SSR markers is more than sufficient to characterize such conversions.

The state of the art is such that it is routine to produce backcross conversions, a statement supporting by Ragot *et al.*, Openshaw *et al.*, as well as basic textbooks on plant breeding. For example, *See* Hallauer *et al.*, "Corn Breeding", Corn and Corn Improvement, No. 18, p. 472 (1988) and Poehlman *et al.*, Breeding Field Crop, 4th Ed., Iowa State University Press, Ames, IA, p. 334 (1995). Specifically, Ragot *et al.* states in the first sentence of the summary "[t]hat molecular markers allow fast recovery of recurrent parent genotype in backcross programs is undisputed", and, in the first sentence of the introduction, "[b]ackcrossing has been a common

breeding practice for as long as elite germplasm has been available." The Applicants' specification teaches that molecular markers of PH8CW can also be used to "reduce the number of crosses back to the recurrent parent needed in a backcrossing program". *See* specification, p. 6, ll. 15-16. In fact, many of the transgenic corn lines currently being commercialized are the result of a backcross conversion of a novel inbred, such as PH8CW.

Similarly, the Examiner's assertion regarding the lack of availability of other inbred lines is also incorrect. There are a multitude of sources from which one may obtain other inbred lines. The National Plant Germplasm System is one such source, and its collection can be easily searched on the World Wide Web at ars-grin.gov/npgs/, where one can also find an on-line request form. A Compilation of North American Maize Breeding Germplasm, Gerdes, J.T., (1993) published by the Crop Science Society of America, has an extensive listing of inbred sources and specific lines available from public universities. Inbred lines are available from the ATCC as a result of deposits made in connection with patents as part of the *quid pro quo* of patent law. Thus, it is clear that contrary to the Examiner's assertion, many inbred lines that may be used as the second inbred line are readily available to the public. One of ordinary skill in the art would certainly know these and other public sources of inbred lines.

Furthermore, Applicants reiterate that the written description requirement of § 112, first paragraph has been fulfilled by depositing seeds of PH8CW in a public depository and by referencing the deposit in the specification. *See* specification, p. 75; *see also Enzo Biochem, Inc. v. Gen-Probe Inc.*, 323 F.3d 956, 965, 63 U.S.P.Q.2d 1609, 1613 (Fed. Cir. 2002) (stating that the written description requirement of § 112, ¶ 1 may be fulfilled by depositing material in a public depository, where the deposited material is not accessible in writing, and where reference to the deposit is made in the specification). This deposit not only describes inbred maize line PH8CW but also the hybrid maize plants, plant parts, and seeds grown in claims 1-12, 15-19, 21-26, and 29-34. In a prior case before the Board of Patent Appeals and Interferences, the Board determined that where claims to an inbred maize plant satisfied the written description requirement, claims to the F1 hybrid seed and plants with the inbred maize plant as a parent also satisfied the written description requirement. *See Ex parte Carlson* (B.P.A.I. 2005). The Board therein stated:

> All that is required by the claims is that the hybrid has one parent that is a plant of corn variety [inbred]. Since the examiner has indicated that the seed and the plant of the corn variety [inbred] are allowable . . . there can be no doubt that the specification provides

> and adequate written description of this corn variety. In addition, the examiner appears to recognize (Answer, page 25) that appellant's specification describes an exemplary hybrid wherein one parent was a plant of the corn variety [inbred]. . . Accordingly, it is unclear to this merits panel what additional description is necessary.

Ex parte Carlson, p. 16. Here, Applicants have done just what the Applicants in *Ex parte Carlson* did, that is claim hybrids having one parent that is a plant of an inbred variety. Further, Applicants reiterates that the specification contains an example of a hybrid produced by PH8CW in the application as filed. *See* specification, p. 52-68, Tables 3A-3F. Thus, under *Ex parte Carlson*, "it is unclear . . . what additional description is necessary." *See Ex parte Carlson*, p. 16; *see also Regents of Univ. of Cal.*, 119 F.3d at 1569, 43 U.S.P.Q.2d at 1406 (stating that an Applicants is "not required to disclose every species encompassed by their claims even in an unpredictable art"). Further, the Examiner has indicated that claim 13, directed towards a plant having all the morphological and physiological traits of PH8CW wherein PH8CW was deposited with the ATCC, is allowable. Accordingly, the genus of hybrid plants and seeds encompassed by claims 1-12, 15-19, 21-26, and 29-34 are as well.

The Examiner further states that "the description of parent inbred line PH8CW and the deposit of PH8CW seed are insufficient to provide adequate written description for seed/plant comprising at least one set of chromosomes of inbred PH8CW or for all hybrid progeny that may be produced by crossing PH8CW plants with any second, distinct corn plants." *See* Office Action, p. 6.

Applicants respectfully traverse. It is correct that "[i]t is well known to one ordinarily skilled in the art that a hybrid made from an inbred will receive one set of chromosomes from that inbred parent" and that "when F1 hybrid seed is produced it will receive one complete set of chromosomes from the inbred parent, regardless of whether the inbred is used as the male or female parent of the F1 hybrid". It is also correct that the "genus of F1 hybrid seed and plants encompassed by Applicant's claims . . . all share the common <u>structural</u> attribute of having a complete or near complete set of the unique chromosomes of PH8CW". Applicants have not stated that all F1 hybrids made with PH8CW would be phenotypically the same. It is true that genetics correlate with phenotype, and that the more highly related two individuals are genetically, the more similar their phenotype is likely to be. It is also true that if one desired to produce an F1 hybrid with the characteristics of the F1 hybrids disclosed in Tables 3A-3E, one of skill in the art would prefer to utilize PH8CW rather than spending the time and resources to

develop a novel inbred. However, the written description requirement does not mandate a description by phenotype. At its foundation, the written description requirement serves an evidentiary function of making certain that the Applicants' are in possession of a specific characteristic that identifies their claimed invention. The molecular marker data provided by Applicants' serves this purpose. The other inbred is not the point of patentability, nor is it what is being claimed. Rather, the claim is drawn precisely to what is described, an F1 hybrid with the identifiable and unique molecular profile of PH8CW.

Lastly, Applicants point out that the legal standards for the written description requirement do not mandate a description via phenotypic characteristics, i.e. plant height, leaf angle, leaf color, etc. Applicants respectfully refer the Examiner to the case of *Ex Parte Tanksley*, 37 U.S.P.Q.2d. 1382. In that case, the Examiner desired that Tanksley claim according to sequence data to "better characterize the cDNA clones" and "facilitate a complete search of the prior art" and issued a § 112 first paragraph written description rejection. The Board held that "the section 112 rejection amounts to a requirement...that the appellants amend their claims in a specified manner...We find no language in the statute or case law which would support that requirement." The Board, in treating the section 112 first paragraph rejection as a § 112 second paragraph rejection, held that "[i]n our judgment, a patent Applicant is entitled to a reasonable degree of latitude in complying with the second paragraph of 35 U.S.C. § 112 and the examiner may not dictate the literal terms of the claims for the stated purpose of facilitating a search of the prior art. Stated another way, a patent Applicant must comply with 35 U.S.C. § 112, second paragraph, but just how the Applicant does so, within reason, is within Applicant's discretion." *Id.* at 1386.

The Examiner states that new claims 11-12, 19 and 21 are rejected because "the SSR loci listed in Table 4 are not structurally or functionally described." *See* Office Action, p. 7.

Applicants respectfully traverse this rejection. As stated *supra*, primers for the SSR markers listed in Table 4 are publicly available as stated in the present application. As explained in the specification, primer sequences for the <u>public</u> SSR markers listed in Table 4 can be easily obtained through the world wide web.

Further, as stated *supra*, Applicants assert that at least 95% of the alleles of inbred line PH8CW disclosed in the SSR profile of Table 4 is an identifying physical characteristic that describes the genus of minor variants of inbred line PH8CW, including, but not limited to, single

locus conversions produced through transformation or introgression. Thus, Applicants again assert one of ordinary skill has been enabled by the deposit to make and use minor variants of inbred corn line PH8CW, and one of ordinary skill in the art uses SSR markers to characterize backcross conversions of an inbred. Applicants have claimed in the manner used by those of ordinary skill in the art to characterize backcross conversions.

The Examiner states that claims 23-25 are rejected because "the claims do not place any limitation on the traits conferred or affected by the single locus conversion," and that the claims "broadly encompass single loci that have not been discovered or isolated." *See* Office Action, p. 7. The Examiner also states that claims 26 are included in the rejection because the specification "provides no description of any plant produced by classical breeding methods such as backcrossing or recurrent selection." *See* Office Action, p. 7.

Applicants respectfully traverse this rejection. The relevant claimed subject matter in claims 23-25 is the plant of claim 13 comprising a transgene or gene conversion. The specification teaches multiple ways of introgressing or transforming a maize plant with various genes which confer advantageous traits desired in the plant. *See* specification, pp. 26-40. The specification also teaches many transgenes that could be inserted into the plant of claim 13. *See* specification, pp. 34-40. Applicants further note that the claims are specifically drawn to a single gene conversion, and that phenotypes resulting from multigenic interactions are not the subject matter of these claims. For example, numerous exemplary transgenes for improved nutritional quality are taught on pages 39-40 of the specification. There are many examples of single gene conversions which affect nutritional quality, see for example, as taught in the specification transforming a plant with an antisense gene of stearoyl-ACP desaturase to increase stearic acid content of the plant, *see* page 39, ll. 17-19, introduction of a phytase-encoding gene that would enhance breakdown of phytate, adding more free phosphate to the transformed plant, *see* page 39, ll. 21-24. In addition, see U.S. Patent No. 5,936,145, issued August 10, 1999, which is prior to the filing date of the instant application. Claim 39 reads as follows: "[t]he single gene conversion of the corn plant of claim 29, where the gene confers enhanced yield stability." Thus, a single gene that confers enhanced yield stability was known in the art prior to the filing date of the instant application. One of skill in the art would recognize that it is common to transform a maize plant with various genes in order to confer desired traits to the maize plant.

The Examiner further states that claims 29-32 are included in the rejection "because the claims read on a method for crossing PH8CW with a multitude of non-exemplified breeding partners which have not been characterized either morphologically or genetically." Claims 33-34 are likewise rejected "because the claims require the use of a multitude of non-exemplified molecular markers." *See* Office Action, p. 8.

Applicants respectfully traverse this rejection. Claims 29-32 and 33-34 are directed towards methods for producing a maize plant derived from PH8CW and developing a maize plant in a plant breeding program where the maize plant of claim 13 is used as a source of breeding material. The language of claims 29-32 and 33-34 makes clear that the maize plant of claim 13 must be used as breeding material in the breeding program described by claims 29-32 and 33-34.

Plant breeding techniques are well known to individuals skilled in the art. The specification describes many of these known techniques. *See* specification, pp. 2-9. In particular, the specification discusses the role of an inbred maize line in a plant breeding program:

> Plant breeding techniques known in the art and used in a maize plant breeding program include, but are not limited to, recurrent selection, backcrossing, pedigree breeding, restriction fragment length polymorphism enhanced selection, genetic marker enhanced selection, making double haploids, and transformation. Often a combination of these techniques are used. The development of maize hybrids in a maize plant breeding program requires, in general, the development of homozygous inbred lines, the crossing of these lines, and the evaluation of the crosses. Maize plant breeding programs combine the genetic backgrounds from two or more inbred lines or various other germplasm sources into breeding populations from which new inbred lines are developed by selfing and selection of desired phenotypes. The new inbreds are crossed with other inbred lines and the hybrids from these crosses are evaluated to determine which of those have commercial potential.

Specification, p. 3, l. 26 through p. 4, l. 3.

As the specification makes clear, one of ordinary skill in the art would know how a maize inbred line is to be used in a plant breeding program. As taught by the specification, the maize inbred is used as a source of germplasm in creating new hybrid lines. It is thus clear from the specification, and to one of ordinary skill in the art, how PH8CW would be employed in a plant breeding program.

Accordingly, Applicants submit that claims 1-12, 15-19, 21-26, and 29-34 are described. In light of the above amendments and remarks, Applicants respectfully request reconsideration and withdrawal of the rejections under 35 U.S.C. § 112, first paragraph.

Rejections Under 35 U.S.C. §§ 102(b)/103(a)

Claims 1-10, 15-18, 22, and 26-34 are rejected under 35 U.S.C. § 102(b) as being anticipated by or, in the alternative, under 35 U.S.C. § 103(a) as obvious over Carlone, Jr., Mario Rosario (U.S. Patent 6,180,857). The Examiner states that "given similar characteristics, the claimed hybrid seed/plant and the prior art hybrid seed/plant are indistinguishable". *See* Office Action, pp. 9-10.

Applicants respectfully traverse this rejection. The Applicants would like to point out that the inventions inbred maize line PH8CW and hybrid maize plant and seed 33P66 are not the same inventions. Carlone does not disclose each of the limitations of claims 1-10, 15-18, 22, and 26-34. Nor are their differences minor morphological variations. Applicants submits that the claimed plant cannot be anticipated by Carlone as inbred maize line PH8CW and hybrid maize plant and seed 33P66 are not the same as it possesses a unique combination of traits which confers a unique combination of genetics. Moreover, Applicants claim a method of making a plant which did not previously exist. Maize inbred line PH8CW has not previously existed as it is the result of crossing two inbred maize lines PH24E and PH1CA (see Appendix 5, PVP Certificate No. 200200187). In contrast Carlone is a hybrid plant that is produced by crossing two inbred maize lines GE492041 with ATCC Deposit PTA-1308 and GE514284 with ATCC Deposit PTA-1518.

Furthermore, when looking at the tables of both inventions, inbreds created using PH8CW as one of the parents are clearly not anticipated by hybrids made using 33P66 as one of the parents. The inventions PH8CW and 33P66 differ for various traits that are not minor. For example:

CHARACTERISTICS	*Inbred PH8CW*	Hybrid 33P66
Comparative Relative Maturity	108	113
Rating System		
Ear Height (cm)	75.3	121.3
Glume Color	Purple	Light Green
Hard Endosperm Color	Pink-Orange	Yellow
Northern Leaf Blight	7	5
Rate from 1 (most susceptible) to 9 (most resistant)		
Gibberella Ear Rot	9	none
Rate from 1 (most susceptible) to 9 (most resistant)		

This comparison clearly shows that PH8CW does not exhibit the characteristics of hybrid 33P66. In addition, it is vital to note that the cited prior art is a hybrid and not an inbred as in the present invention and one of ordinary skill in the art would know the major differences between a hybrid and inbred. The aforementioned examples all illustrate that there are large differences between PH8CW and 33P66. The examples listed are not exhaustive but they do give ample evidence that the inventions are not the same. Furthermore, when looking at the tables of both inventions, plants created using PH8CW as one of the parents are clearly not anticipated by hybrids made using 33P66 as one of the parents.

When looking at a maize plant it would be possible to find many traits that are similar between varieties such as the color of flowers or growth habit. However, to say there are similarities in phenotype between two varieties is not the same as saying that the two varieties have the same morphological and physiological characteristics as a whole, or that one is an obvious variant of the other.

As described *supra*, inbred maize line PH8CW does not exhibit the same characteristics as hybrid maize plant and seed 33P66. The Examiner has not provided any reference that may be combined with 33P66 to arrive at the present invention. The Examiner has not provided a single reference with all elements of the claimed invention, nor a reference that could be

combined with the Carlone patent to produce PH8CW. Applicants respectfully assert that a prima facie case of obviousness has not been made, and reconsideration is respectfully requested. Thus, Applicants submit that the claimed plant cannot be rendered obvious over Carlone. Inbred PH8CW deserves to be considered as a new and non-obvious composition in its own right as does its products of the process when Inbred PH8CW is used as starting material. Applicants point out that PH8CW is a unique inbred plant which never before existed until Applicants filed the application and until its deposit of the same.

Therefore, Carlone does not teach the seed or plant of PH8CW, or an F1 seed or plant produced from PH8CW. Therefore, because Carlone does not teach PH8CW, it can not anticipate nor is it obvious over claims 1-10, 15-18, 22, and 26-34.

In light of the above, Applicants respectfully request the Examiner reconsider and withdraw the rejections to claims 1-10, 15-18, 22, and 26-34 under 35 U.S.C. § 102(b) or 35 U.S.C. § 103(a) as obvious over Carlone, Jr., Mario Rosario (U.S. Patent 6,180,857).

Request for Information under 37 C.F.R. § 1.105

The Examiner has made a Request for Information under 37 C.F.R. § 1.105. The Examiner states the requested information is "required to make a meaningful and complete search of the prior art". *See* Office Action – Request for Information Under 37 C.F.R. § 1.105, p. 2.

Applicants provide answers to each of the Examiner's interrogatories discussed *infra.*

The Examiner begins by asking firstly, what were the original parental maize lines used to produce maize inbred line PH8CW? Please supply information pertaining to the lineage of the original parental lines back to any publicly available varieties. PH24E and PH1CA. Information pertaining to the lineage of the original parental lines is available within the PVP Application No. 200200187, attached as Appendix 5.

Secondly, what method and steps were used to produce maize inbred line PH8CW? Pedigree selection method produced by selfing for 7 generations.

Third, have any of said parental maize lines or progeny therefrom been disclosed or made publicly available?

a. The parental maize line PH24E was previously disclosed or made publicly available in PVP Certificate No. 9600204 and U.S. Patent No. 5,689,034. The parental maize line PH1CA

was previously disclosed or made publicly available in PVP Certificate No. 9800386 and U.S. Patent No. 6,025,547.

b. No other progeny of the parental cross PH24E/PH1CA was previously disclosed or made publicly available by Applicant prior to the earliest priority date.

Fourth, were any other maize lines produced by said method using said original parental maize lines, and if so, have said produced maize lines been publicly available or sold? If so, under what designation/denomination and under what conditions were said other maize lines disclosed or made publicly available? No other maize line using the same F1 cross has been produced by said method using said original parental maize lines at or before the time of filing of the instant application.

In light of the above remarks, Applicants respectfully request reconsideration and compliance with the interrogatories under the Request for Information under 37 C.F.R. § 1.105.

Conclusion

In conclusion, Applicants submit in light of the above amendments and remarks, the claims as amended are in a condition for allowance, and reconsideration is respectfully requested. If it is felt that it would aid in prosecution, the Examiner is invited to contact the undersigned at the number indicated to discuss any outstanding issues.

No other fees or extensions of time are believed to be due in connection with this amendment; however, consider this a request for any extension inadvertently omitted, and charge any additional fees to Deposit Account No. 26-0084.

Reconsideration and allowance is respectfully requested.

Respectfully submitted,

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Marker-assisted backcrossing: a practical example

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Summary

That molecular markers allow fast recovery of recurrent parent genotype in backcross programs is undisputed. Restriction Fragment Length Polymorphisms (RFLP's) were used in maize to introgress by backcross a transgene construct, containing phosphinothricin resistance and insecticidal protein genes, from a transformed parent into an elite inbred line. At each generation plants carrying the transgene construct were selected based on their phosphinothricin resistance, and further characterized with RFLP's. Both maximum recovery of recurrent parent genotype and minimum linkage drag were taken into account for marker-based selection. Embryo rescue was used to shorten generation time. Progress towards recurrent parent genotype was spectacular. Levels of recurrent parent genotype recovery which would normally be observed, in the absence of selection, in the BC_6 generation were obtained at the BC_3 generation, about one year after BC_1 seeds had been planted. Besides the evidence already provided by RFLP's, phenotypic evaluation of the backcross-derived near-isogenic lines will constitute an additional check of the completeness of the conversion.

Introduction

Backcrossing has been a common breeding practice for as long as elite germplasm has been available. It has mainly been used to introgress single Mendelian traits, such as disease resistances or quality factors, into elite germplasm (Allard 1960; Hallauer and Miranda 1981). One of the most attractive attributes of backcrossing is that it allows to perform targeted modifications without disrupting the existing overall genetic balance of the recurrent parent.

However, production of fully converted near isogenic lines through classical backcrossing procedures is a lengthy procedure, if at all possible. Theoretically, a minimum

APPENDIX 1

of seven classical backcross generations are required to recover more than 99% of recurrent parent genotype, assuming no linkage drag. The attractiveness of classical backcross procedures is therefore substantially diminished for crops, such as maize (*Zea mays* L.), where the turn-over of elite cultivars is very fast. In addition, full recovery of recurrent parent genotype is usually not achieved through classical backcrossing, which may result in deleterious agronomic effects. Murray *et al.* (1988) reported about 90% recurrent parent genotype recovery in two BC_{10}-equivalent conversions (A632Ht and A632Rp) of the maize line A632. The conversions had retained respectively 4 and 7 donor fragments in addition to the one carrying the gene of interest.

Reduction in the number of backcross generations needed to obtain fully converted individuals has been shown theoretically, or from simulations, to be achievable through the use of molecular markers (Tanksley *et al.* 1989; Hospital *et al.* 1992; Jarboe *et al.* 1994). Because they provide thorough characterization of the genetic variability at each backcross generation, markers allow to take full advantage of this variability by applying the highest possible selection intensity.

Efficiency of marker-assisted backcrossing was investigated through an experiment aimed at introgressing a single genetic factor (a transgene construct) from a donor into a recipient maize line.

Materials and methods

Plant Material

A hemizygous transgenic maize line of Lancaster origin was used as donor parent to introgress its transgene construct, through repeated backcrossing, into a recipient parent from the Stiff Stalk germplasm group. Both parents are proprietary elite lines. The transgene construct carries both a phosphinothricin resistance gene and synthetic genes encoding the entomotoxic fragment of the CryIA(b) *Bacillus thuringiensis* protein (Koziel *et al.* 1993). Transformation was achieved through microprojectile bombardment (Koziel *et al.* 1993) and resulted in a single insertion (*Bt* locus), on chromosome 1 (Figure 1).

Backcross protocol

The F1 progeny of the cross between the donor and the recipient was screened for the presence of the transgene construct by applying Basta, a phosphinothricin-based herbicide, onto each plant. Resistant individuals were then used to generate BC_1 progeny.

For each backcross generation, except the BC_4, individuals were planted in multipots and sprayed with Basta to eliminate those which did not carry the transgene construct. To avoid the stress resulting from treatment with Basta, BC_4 plants carrying the transgene construct were identified using Southern blots probed with the *pat* and *Bt* genes. Resistant plants were transplanted in an open-soil greenhouse and leaf-sampled for molecular marker

analyses. Results of marker an
flowering. A single plant was
rescued and transferred onto ti
embryos first underwent a gre
culture medium, before being
average, four months.

Molecular marker analys

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chemiluminescent techniques. I
were chosen from among those :
provided coverage of the entire
contained two loci tightly linked
recombination units away (Figu
BC_{n+1} generation comprised bo
or tightly linked ones, and addi
selected BC_n plant was heterozy
independent reference populati
generation.

Selection procedure

At each generation plants
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for the BC_3 selection) was avail

Results and discussion

Selection for the gene o

The observed segregation
significantly different ($P<0.05$

Recurrent parent genoty

Statistics for the genoty
performed taking the whole g
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analyses. Results of marker analyses were made available at the latest two weeks after flowering. A single plant was selected, of which all backcross-derived embryos were rescued and transferred onto tissue culture medium. Plantlets that developed from these embryos first underwent a greenhouse acclimation phase, while still growing on tissue culture medium, before being transplanted into multipots. Backcross cycles lasted, on average, four months.

Molecular marker analyses

Restriction Fragment Length Polymorphisms (RFLP's) were used to establish genotypes in all four generations. RFLP detection involved either radioactive or chemiluminescent techniques. For the BC_1 generation, 61 marker-enzyme combinations were chosen from among those revealing polymorphism between donor and recipient. They provided coverage of the entire genome, defining intervals of about 25 cM in size, and contained two loci tightly linked to the *Bt* locus, CG320 and CG415, respectively 5 and 16 recombination units away (Figure 1). For subsequent generations, markers analyzed in the BC_{n+1} generation comprised both those for which the selected BC_n plant was heterozygous, or tightly linked ones, and additional ones located in chromosomal segments for which the selected BC_n plant was heterozygous (Table 1). Marker map positions were obtained from independent reference populations and confirmed by analysis of segregation in the BC_1 generation.

Selection procedure

At each generation plants were ranked based both on the percentage of homozygous recurrent-parent-genotype and on the extent of linkage drag around the *Bt* locus, in an attempt to integrate both criteria. Plants for which two or more adjacent markers had missing values were not included in the analyses. Success or failure of the pollinations also contributed to the selection procedure. One single plant was selected at each generation: the best ranking one of those for which a backcross progeny of size 100 or more (50 or more for the BC_3 selection) was available.

Results and discussion

Selection for the gene of interest

The observed segregation ratios for phosphinothricin resistance (Table 1) were not significantly different ($P<0.05$) from the expected 1:1, as shown by Chi-square tests.

Recurrent parent genotype recovery

Statistics for the genotyped plants are summarized in Table 1. Calculations were performed taking the whole genome into account, including the *Bt* locus. The "perfect" backcross-derived plant therefore counts one heterozygous chromosome segment, that

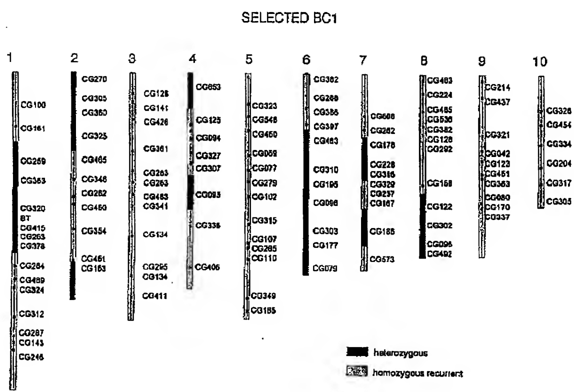
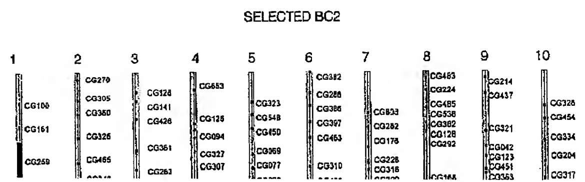


Figure 1-a: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.



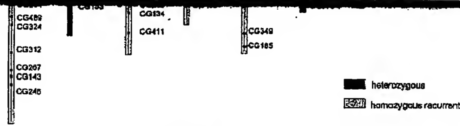


Figure 1-a: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

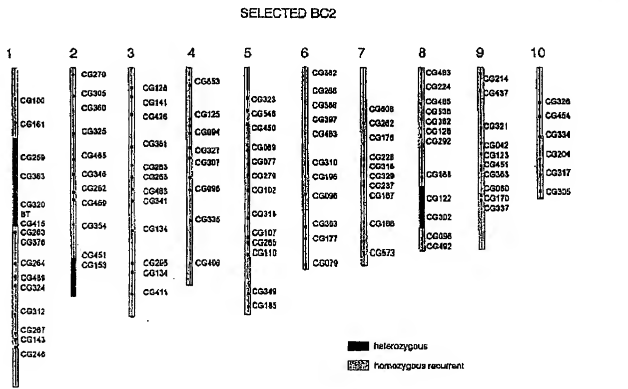


Figure 1-b: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

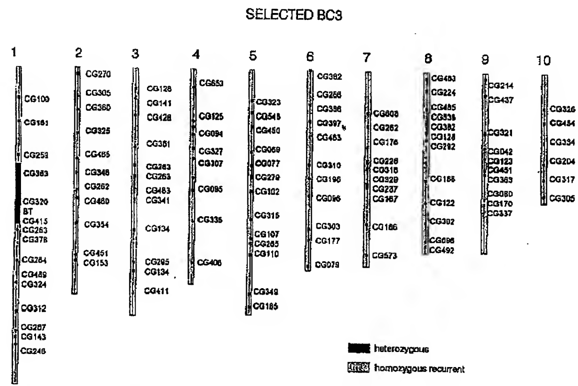
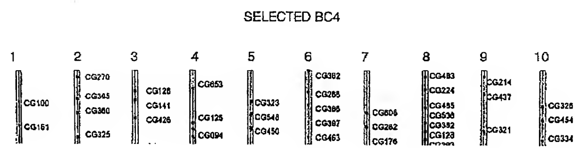


Figure 1-c: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.



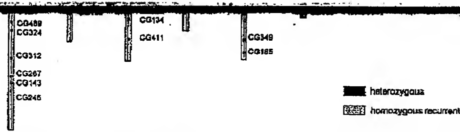


Figure 1-c: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

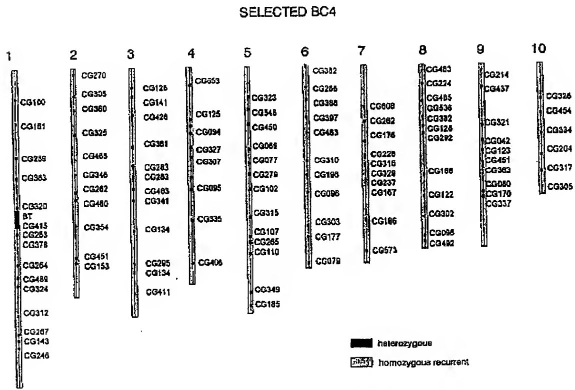


Figure 1-d: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

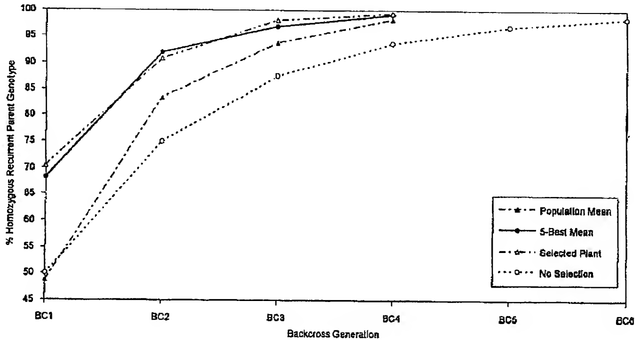


Figure 2: Recovery of recurrent parent genotype through backcrossing, with or without marker-assisted selection

Table 1: Proportion and characteristics of plants carrying the genes of interest, in the first four generations of a marker-assisted backcross program.

generation	% phosphinothricin	RFLP genotyping	nb plants	% homozygous recurrent	nb heterozygous

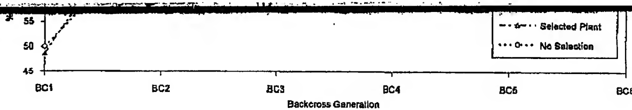


Figure 2: Recovery of recurrent parent genotype through backcrossing, with or without marker-assisted selection

Table 1: Proportion and characteristics of plants carrying the genes of interest, in the first four generations of a marker-assisted backcross program.

generation	% phosphinothricin resistant plants	RFLP genotyping			nb plants analyzed *	% homozygous recurrent parent genotype				nb heterozygous chromosome segments ***			
		nb plants	nb loci	nb datapoints		mean	std dev	5-best mean **	selected plant	mean	std dev	5-best mean **	selected plant
BC1	49.05	96	61	5656	87	48.72	10.35	88.31	70.45	11.01	2.17	7.75	6
BC2	44.65	61	22	1342	30	83.42	5.64	91.98	90.84	5.03	1.54	3.20	3
BC3	46.32	72	10	720	71	93.83	1.85	96.82	98.03	2.20	0.71	1.60	1
BC4	-	26	3	78	26	98.23	0.49	99.09	99.36	1.00	0.00	1.00	1

* Plants for which two or more adjacent markers had missing values were not included in the analyses
** Mean value of the five individuals having the five highest percentages of homozygous recurrent parent genotype.
*** Including the segment carrying the transgene construct.

comprising the *Bt* locus. It also displays 99.36% of homozygous recurrent-parent-genotype. The remaining 0.64% corresponds to the average relative length of the chromosome segment containing the *Bt* locus, which depends on the two flanking markers chosen.

The mean percentage of homozygous recurrent-parent-genotype of the BC_1 generation was slightly lower than the expected 50%. This can be explained by linkage drag around the *Bt* locus, given that this percentage was computed based only on plants selected for heterozygosity at the *Bt* locus. For all other backcross generations the mean percentage of homozygous recurrent-parent-genotype was much higher than what would have been observed, should no selection have been done (Figure 2).

The percentage of homozygous recurrent-parent-genotype of the selected plant (Table 1) and the average of the five largest values (Table 1) were always very similar to one another, and much superior to the population mean value (Figure 2). The percentage of homozygous recurrent-parent-genotype of the selected plant was found only once, in the BC_2 generation, to be smaller than the average of the five largest values. This corresponded to the only time when the selected plant was not the one with the maximum percentage of homozygous recurrent-parent-genotype. The plant had been selected because it displayed a favorable recombination on one side of the *Bt* locus (Figure 1).

The percentage of homozygous recurrent-parent-genotype of the selected BC_1 plant was almost equal to that of an unselected BC_2, that of the selected BC_2 was larger than that of an unselected BC_3, that of the selected BC_3 was barely smaller than that of an unselected BC_6, and that of the selected BC_4 was equal to that of the "perfect" backcross-derived plant, given the set of markers that was used. Such rates of recurrent parent genotype recovery are consistent with results of simulation analyses. Jarboe *et al.* (1994) who used the maize genome as a model reported that three backcross generations and 80 markers were needed to recover 99% of recurrent parent genotype.

Number of donor chromosome segments

The number of heterozygous chromosomal segments decreased from one backcross generation to the next. Plants selected at each generation were not necessarily those which had the lowest number of heterozygous chromosomal segments (Table 1). However, with the set of markers used, BC_3 and BC_4 plants were recovered which contained only one heterozygous chromosomal segment: that comprising the *Bt* locus.

Linkage drag

Linkage drag around the *Bt* locus was estimated, relative to the length of chromosome 1. Its value was found to lie between 24.0 and 48.4% for the selected BC_1 individual, between 17.6 and 34.8% for the selected BC_2, between 2.0 and 24.0% for the selected BC_3, and between 0.0 and 8.4% (respectively 0.0 and 14.5 cM) for the selected BC_4.

The two values given for each ge
correspond to extreme positions o
flanking the transgene construct locu
BC_4 is likely to be less than 1.3%
appear to be somewhat high, reflec
drag, it is much lower than what
(Stam and Zeven 1981; Tanksley *et*
of tomato cultivars obtained by a l
Tanksley (1989) found that the sizes
cM.

Conclusion

These results clearly demonstr
quality advantages over classical
through backcrossing. Only four ba
than a year and a half from plan
genotypically fully converted. Neve
genotype could proceed even faste
appropriate protocol and resources
allocated.

Comparison of BC_4-derived
markers and agronomic performanc
order to confirm the completeness o

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The two values given for each generation are extreme values of linkage drag, which correspond to extreme positions of the crossing-overs in the marker-defined intervals flanking the transgene construct locus. Therefore the true linkage drag value of the selected BC_4 is likely to be less than 1.3% of the genome. Although this maximum value may appear to be somewhat high, reflecting the limited selection pressure put here on linkage drag, it is much lower than what would be expected from classical backcross programs (Stam and Zeven 1981; Tanksley *et al.* 1989). Practically, in a study of *Tm*-2 conversions of tomato cultivars obtained by a large number of classical backcross cycles, Young and Tanksley (1989) found that the sizes of the introgressed fragments ranged between 4 and 51 cM.

Conclusion

These results clearly demonstrate that molecular markers provide important time and quality advantages over classical procedures for the production of near-isogenic lines through backcrossing. Only four backcross generations were necessary to recover, in less than a year and a half from planting of the BC_1's, individuals which appeared to be genotypically fully converted. Nevertheless, it is likely that recovery of recurrent parent genotype could proceed even faster than in the experiment described herein, should the appropriate protocol and resources (population size, number and position of markers) be allocated.

Comparison of BC_4-derived lines with the recurrent parent for both morphological markers and agronomic performance (including hybrid performance) will be performed in order to confirm the completeness of the conversion.

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Marker-assisted Selection in Backcross Breeding

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Abstract. **The backcross breeding procedure has been used widely to transfer simply inherited traits into elite genotypes. Genetic markers can increase the effectiveness of backcrossing by 1) increasing the probability of obtaining a suitable conversion, and 2) decreasing the time required to achieve an acceptable recovery. Simulation and field results indicated that, for a genome consisting of ten 200-cM chromosomes, basing selection on 40 or 80 markers in 50 BC individuals that carry the allele being transferred can reduce the number of backcross generations needed from about seven to three.**

The backcross breeding procedure has been used widely to transfer simply inherited traits into elite genotypes. Usually, the trait being transferred is controlled by a single gene, but highly heritable traits that are more complexly inherited have also been transferred successfully by backcrossing; for example, maturity in maize (Rinke and Sentz, 1961; Shaver, 1976). Today, backcrossing is being used to transfer genes introduced by such techniques as transformation or mutation into appropriate germplasm.

Several plant breeding textbooks give good descriptions of the backcross procedure (Allard, 1960; Fehr, 1987). A donor parent (DP) carrying a trait of interest is crossed to the recurrent parent (RP), an elite line that is lacking the trait. The F_1 is crossed back to the RP to produce the BC_1 generation. In the BC_1 and subsequent backcross generations, selected individuals carrying the gene being transferred are backcrossed to the RP. The expected proportion of DP genome is reduced by half with each generation of backcrossing. Ignoring effects of linkage to the selected DP allele being transferred, the percentage recurrent parent (%RP) genome expected in each backcross generation is calculated as:

$$\%RP = 100\,[1 - (0.5)^{n+1}]$$

where n is the number of backcrosses.

Backcrossing of selected plants to the RP can be repeated each cycle until a line is obtained that is essentially a version of the RP that includes the introgressed allele. After six backcrosses, the expected recovery is >99% (Table 1).

Until recently, discussions of the recovery of the RP genome during backcrossing have emphasized the expected values for %RP shown in Table 1, and have largely ignored the genetic variation for %RP that exists around the expected mean. With the development of genetic markers capable of providing good genome coverage, there has been interest in taking advantage of that variation to increase the efficiency of backcrossing.

Selection for RP marker alleles can increase greatly the effectiveness of backcross programs by allowing the breeder to 1) select backcross plants that have a higher proportion of RP genome, and 2) select backcross individuals that are better conversions near a mapped donor allele being transferred (i.e., select for less linkage drag). Expressed in practical terms, using genetic markers to assist backcrossing can 1) increase the probability of obtaining a suitable conversion, and 2) decrease the time required to achieve an acceptable recovery.

Issues to consider when planning a marker-assisted backcross program include 1) the time advantage of using markers to assist backcrossing, 2) the number of markers needed, and 3) the number of genotypes to evaluate. In this report, we use results from previous literature, computer simulation, and empirical studies to provide some guidelines.

Table 1. *Expected recovery of recurrent parent (RP) genome during backcrossing, assuming no linkage to the gene being transferred.*

Generation	%RP
F_1	50.0000
BC_1	75.0000
BC_2	87.5000
BC_3	93.7500
BC_4	96.8750
BC_5	98.4375
BC_6	99.2188
BC_7	99.6094

[1]Formerly with Purdue University, West Lafayette, Ind.

APPENDIX 2

Materials and methods

The maize genome was the model for the simulation. The simulated genome contained ten 200-cM chromosomes. Simulation of crossing over was based on a Poisson distribution with a mean of 2.0 ($\lambda = 2$) (Hanson, 1959), which, on average, generated one cross over for every 100-cM length. The simulations reported here assume no interference. Codominant genetic markers were evenly distributed in the genome and sites of the donor gene were randomly assigned to genome locations.

Simulations were conducted with the following parameters:

Number of progeny: 100 or 500.
Backcross generations: BC_1, BC_2, and BC_3.
Number of markers: 20, 40, 80, or 100.
Number selected to form the next BC generation: 1 or 5.

Selection was based on 1) presence of the donor allele and 2) high %RP). %RP was calculated as the average of the (one or five) selected individuals. Values presented are the mean of 50 simulations.

Results

In the computer simulation study, all methods modeled greatly increased the speed of recovering the RP genome compared to the expected recovery with no marker-assisted selection (compare Tables 1 and 2). At least 80 markers were required to recover 99% of the RP genome in just three BC generations (Table 2). Use of at least 80 markers and 500 progeny allowed recovery of 98% RP in just two BC generations. Response to selection was diminished only slightly by spreading the effort over five selections. Using markers, the number of backcross generations needed to convert an inbred is reduced from about seven to three.

By the BC_3 generation, there appears to be no practical advantage to using 500 vs. 100 individuals. If the presence of the donor trait in the backcross individuals can be ascertained before markers are genotyped, then only half the number of individuals indicated in the tables will need to be analyzed.

When a small number of markers are used, they quickly became non-informative; i.e., selection causes the marker loci to become fixed for the RP type before the rest of the genome is fully converted (Table 3; Hospital et al., 1992). This situation was most prominent in the larger populations, where a higher selection intensity placed more selection pressure upon the marker loci. Accordingly, it is of interest to consider how closely the estimation of %RP based on markers reflects the actual genome composition. The combination of estimation of %RP based on fewer markers and subsequent selection tends to bias the estimates upward (compare Tables 2 and 3).

The results from the simulation compare well with real field data. In a typical example, 50 BC_1 plants carrying the gene being transferred were genotyped at 83 polymorphic RFLP loci (note that this corresponds to a population size of 100 unselected plants in Tables 2 and 3). The five best BC_1 recoveries had estimated %RP values of 85.9%, 82.7%, 82.0%, 81.4%, and 81.2%. After evaluating 10 BC_2 plants from each selected BC_1, the best BC_2 recovery had an estimated %RP of 94.6%.

Discussion

The simulations (Table 2; Hospital et al., 1992) and our experience indicate that four markers per 200-cM chromosome is adequate to greatly increase the effectiveness of selection in the BC_1. However, using only four markers per 200 cM will likely make it very difficult to map the location of the gene of interest. Adequate summarization of the data is an important

Table 2. *Percent recurrent parent genome during marker-assisted backcrossing.*

Generation	100 Progeny No. markers 20	40	80	100	500 Progeny No. markers 20	40	80	100
				One selected				
BC_1	84.5	84.5	84.2	88.0	89.9	90.7	90.2	90.5
BC_2	95.0	95.2	95.8	97.2	96.5	97.7	98.5	98.6
BC_3	97.4	97.6	98.9	99.2	97.7	98.3	99.4	99.5
				Five selected				
BC_1	82.9	85.1	84.9	84.7	87.7	88.1	88.9	88.9
BC_2	93.7	95.0	95.8	95.7	95.5	96.8	97.8	97.9
BC_3	97.1	98.3	98.8	98.9	97.3	98.5	99.3	99.3

Table 3. *Estimates of percent recurrent parent genome, based on marker loci.*

Generation	100 Progeny No. markers 20	40	80	100	500 Progeny No. markers 20	40	80	100
				One selected				
BC_2	98.7	97.8	95.6	97.2	100.0	99.1	98.6	98.0
BC_3	100.0	99.8	99.3	99.5	100.0	100.0	99.9	98.2
				Five selected				
BC_2	96.4	96.5	96.2	95.8	100.0	98.5	98.3	98.2
BC_3	99.9	99.8	99.3	99.1	100.0	100.0	99.9	99.8

part of a marker-assisted backcross program. Ideally, the markers used can supply data that can be represented as alleles of loci with known map position. Estimation of %RP, mapping the position of the locus of interest, and graphical display of the results (Young and Tanksley, 1989) are all useful in understanding and controlling the specific backcross experiment being conducted.

It appears that, with the use of genetic markers, the portion of the RP genome that is not linked to the allele being transferred can be recovered quickly and with confidence. The recovery of RP will be slower on the chromosome carrying the gene of interest. A considerable amount of linkage drag is expected to accompany selection for the DP allele in a backcross program. For a locus located in the middle of a 200-cM chromosome, the length of the DP chromosome segment accompanying selection is expected to be 126, 63, and 28 cM in the BC_1, BC_3, and BC_7 generations, respectively (Hanson, 1959; Naveira and Barbadilla, 1992). Our observations support the recommendation of Hospital et al. (1992) that preference be given to the selection for recombinants proximal to the allele of interest, but that selection for recovery of the RP elsewhere in the genome also be considered. This two-stage selection can probably be done quite effectively ad hoc by the breeder once the data is adequately summarized; however, Hospital et al. suggest ways to incorporate the two criteria into a selection index such that each component of selection is assured appropriate weighting.

Use of genetic markers can greatly increase the effectiveness of backcrossing, and they should be used in any serious backcrossing program if resources are available to the breeder.

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bnlg1014 (locus)

This locus is also known by the following names:
bmc1014
bngl1014

Type: Probed Site
Species: Zea mays ssp. mays
Linkage Group: 1
Arm: S (short arm)

Map Coordinates: (* indicates the locus is on the backbone)

Map	Coordinate	Bin
A632/rtcs1 1999	20.00	1.01
bins 1	1.01	1.01
BNL 2002 1	41.68	1.01
Chromatin IBM 2003 1 *	82.80	1.01
IBM IDP +MMP bd (ver 4) 1	48.91	1.01
IBM neighbors v.2 1 *	76.40	1.01
IBM1 1 *	76.40	1.01
IBM2 1 *	82.80	1.01
IBM2 2004 neighbors 1 *	82.80	1.01
IBM2 2004 neighbors frame 1 *	82.80	1.01
IBM2 FPC0402 genetic neighbors 1 *	83.02	1.01
IBM2 neighbors 1 *	82.80	1.01
IBM2 neighbors frame 1 *	82.80	1.01
LHRF Gnp2004 1 *	16.00	
Pioneer composite 1999 1	20.70	1.01
SSR Consensus 1	24.50	1.01
SSR IBM 1 *	66.10	1.01
SSR Tx303xCO159 2002 1 *	21.90	1.01
SSR Tx303xCO159 2003 1 *	22.00	1.01

SSRs
p-bnlg1014 (via SSR PCR)

Primers and Enzymes:

Primer/Enzyme	Probe
CACGCTGTTTCAGACAGGAA	p-bnlg1014
CGCCTGTGATTGCACTACAC	p-bnlg1014

Anchored BACs: (BACs identified to be anchored by probes for this locus):

b0074A07	b0092D02	b0138L14	b0008N23
b0036L14	b0182A10	b0284B14	

APPENDIX 3

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An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays L.*): comparisons with data from RFLPS and pedigree

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Abstract The utility of 131 simple sequence repeat (SSR) loci to characterize and identify maize inbred lines, validate pedigree, and show associations among inbred lines was evaluated using a set of 58 inbred lines and four hybrids. Thirteen sets of inbred parent-progeny triplet pedigrees together with four hybrids and their parental lines were used to quantify incidences of scoring that departed from expectations based upon simple Mendelian inheritance. Results were compared to those obtained using 80 restriction fragment length polymorphism (RFLP) probes. Over all inbred triplets, 2.2% of SSRs and 3.6% of RFLP loci resulted in profiles that were scored as having segregated in a non-Mendelian fashion. Polymorphic index content (PIC, a measure of discrimination ability) values ranged from 0.06 to 0.91 for SSRs and from 0.10 to 0.84 for RFLPs. Mean values for PIC for SSRs and RFLPs were similar, approximately 0.62. However, PIC values for nine SSRs exceeded the maximum PIC for RFLPs. Di-repeats gave the highest mean PIC scores for SSRs but this class of repeats can result in "stutter" bands that complicate accurate genotyping. Associations among inbreds were similar for SSR and RFLP data, closely approximating expectations from known pedigrees. SSR technology presents the potential advantages of reliability, reproducibility, discrimination, standardization and cost effectiveness over RFLPs. SSR profiles can be readily interpreted in terms of alleles at mapped loci across a broad range of maize germ plasm. Consequently, SSRs represent the optimum approach for the identification and pedigree validation of maize genotypes compared to other currently available methods.

Key words Simple sequence repeat · Microsatellite · SSRs · Maize · Variety identification

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Introduction

Microsatellites, or simple sequence repeats (SSRs) are short nucleotide sequences, usually from 2 to 3 bases(b) in length that are repeated in tandem arrays. Amplifiable polymorphisms are revealed because of differences in the numbers of tandem repeats that lie between sequences that are otherwise conserved for each locus. Microsatellite loci have proven to be highly polymorphic and useful as genetic markers in many plant species including *Arabidopsis* (Depeiges et al. 1995), bur oak (Dow et al. 1995), maize (Senior and Heun 1993), seashore paspalum (Liu et al. 1995), rapeseed (Kresovich et al. 1995; Charters et al. 1996), soybean (Akkaya et al. 1992, 1995; Rongwen et al. 1995), sugar beet (Mörchen et al. 1996), sweet potato (Jarret and Bowen 1994) and wheat (Plaschke et al. 1995; Roder et al. 1995).

In this paper, we report the usefulness of SSRs as genetic markers to discriminate between, and to show associations among, inbred lines of maize using a greater number of loci and a broader diversity of maize germ plasm than has been reported previously (Senior and Heun 1993).

Appendix 4

Table 1 List and pedigree background of inbred lines used in the present SSR and RFLP profiling study

A632	Pedigree background[a]
A632	BSSS[b] C0 (94%), Minesota 13[c] (6%)
B73	BSSS[b] (100%)
Mo17	Lancaster Sure Crop[c] (50%), Krug[c] (50%)
PH207	Iodent[c] (59%), Long Ear[c] (20%), Minesota 13[c] (11%), Troyer Reid[c] (5%)
B64	BSSS[b] C0 (87.5%), Maiz Amargo[c] (12.5%)
PH595	Midland Yellow Dent[c] (25%), Southern U.S. Landrace Synthetic (19%), Funks G4949 (12.5%), Illinois Long Ear[c] (12.5%), Illinois Two Ear (12.5%)
PH642	BSSS[b] C0 (87.5%), Iodent[c] (9%)
PH814	Lancaster Low Breakage (25%), Southern U.S. Landrace Synthetic (19%), Osterland Yellow Dent[c] (16%), Funks G4949 (13%), Midland Yellow Dent[c] (6%), Tuson B[d] (6%), Brookings 86[c] (5%)
PH848	Minnesota 13[c] (12.5%), Osterland Yellow Dent[c] (12.5%), SRS303[cf] (12.5%), Iodent[c] (12%), Reid Yellow Dent[c] (12%), Lancaster Sure Crop[c] (6%), Longfellow Flint[c] (6%), MHW[d] (6%)
PHB09	BSSS[b] C0 (62.5%), Minnesota 13[c] (25%)
PHB46	BSSS[b] C0 (50%), Alberta Flint[c] (25%), Osterland Yellow Dent[c] (25%)
PHB47	BSSS[b] C0 (87.5%), Brookings 86[c] (12.5%)
PHB76	Smith TC[h] (25%), Midland Yellow Dent[c] (12.5%), NW Dent[i] (12.5%), Southern U.S. Landrace Synthetic (9%), Minnesota 13[c] (8%), Funks G4949 (6%), Illinois Long Ear (6%), Illinois Two Ear[c] (6%), Osterland Yellow Dent[c] (6%)
PHB89	Coker 616 (25%), Lancaster Sure Crop[c] (12.5%), Midland Yellow Dent[c] (12.5%), Southern U.S. Landrace Syndrace Synthetic (9%), Minesota 13[c] (8%), Funks G4949 (6%), Funks Yellow Dent[c] (6%), Illinois Long Ear[c] (6%), Illinois Two Ear (6%)
PHBE2	Iodent[c] (18%), Southern U.S. Landrace Synthetic (9%), Minnesota 13[c] (9%), Osterland Yellow Dent[c] (6%), Midland Yellow Dent[c] (6%), Long Ear (6%), Funks G4949 (6%), Lancaster Low Breakage (5%)
PHBG4	Iodent[c] (27%), Minnesota 13[c] (11%), Long Ear (9%), Coker 616 (8%), Midland Yellow Dent[c] (6%), Lancaster Sure Crop[c] (6%), Southern U.S. Landrace Synthetic (6%)
PHG12	BSSS[b] C0 (37.5%), Lancaster Low Breakage (25%), M3204[j] (25%)
PHG29	Iodent[c] (59%), Long Ear (20%), Minnesota 13[c] (13%), Troyer Reid[c] (5%)
PHG31	Iodent[c] (44%), Long Ear (15%), Minnesota 13[c] (11%), Midland Yellow Dent[c] (6%), Southern U.S. Landrace Synthetic (5%)
PHG35	Iodent[c] (29%), Midland Yellow Dent[c] (13%), Minnesota 13[c] (11%), Southern U.S. Landrace Synthetic (9%), Long Ear (9%), Funks G4949 (6%), Illinois Long Ear (6%), Illinois Two Ear (6%)
PHG39	BSSS[b] C0 (69%), Maiz Amargo[c] (25%)
PHG42	Iodent[c] (30%), Lancaster Low Breakage (10%), Southern U.S. Landrace Synthetic (9%), Osterland Yellow Dent[c] (9%), Minnesota 13[c] (7%), Funks G4949 (6%)
PHG45	Iodent[c] (59%), Long Ear (20%), Minnesota 13[c] (13%), Troyer Ried[c] (5%)
PHG50	Iodent[c] (35%), Long Ear (12%), Minnesota 13[c] (12%), Osterland Yellow Dent[c] (7%), SRS 303[c] (6%), Reid[c] (6%)
PHG53	BSSS[b] C0 (91%), Maiz Amargo[c] (6%)
PHG55	PROCOMP[k] (50%), Minnesota 13[c] (6%), Osterland Yellow Dent[c] (6%), SRS 303[c] (6%), Iodent[c] (6%), Reid[c] (6%)
PHG69	BASS[b] (50%), BSSS[b] (50%) BSSS[b] C0 (25%), Alberta Flint (13%), Osterland Yellow Dent[c] (13%)
PHG71	BSSS[1]C0 (47%), Iodent[c] (30%), Long Ear (10%), Minnesota 13[c] (9%)
PHG74	BSSS[b] C0 (89%), Minnesota 13[c] (5%)
PHG80	Dockendorf 101[c] (50%), BSSS[b] C0 (38%)
PHG81	BSSS[b] (50%), Iodent[c] (30%), Long Ear (10%), Minnesota 13[c] (6%)
PHG83	Iodent[c] (30%), Lancaster Low Breakage (13%), Long Ear (10%), Southern U.S. Landrace Synthetic (9%), Osterland Yellow Dent[c] (9%), Minnesota 13[c] (7%), Funks G 4949 (6%)
PHG84	Midland Yellow Dent[c] (13%), Southern U.S. Landrace Synthetic (9%), Minnesota 13[c] (8%), Funks G4949 (6%), Illinois Low Ear (6%), Illinois Two Ear (6%), Osterland Yellow Dent[c] (6%), SRS 303[i] (6%), Iodent[c] (6%), Reid[c] (6%)
PHG86	BSSS[b] (50%), BSSS[b] C0 (44%), Maiz Amargo[c] (6%)
PHJ76	BSSS[b] (50%), BSSS[b] C0 (38%)
PHK29	BSSS[b] C0 (63%), BSSS[b] (25%), Brookings 86[c] (6%)
PHK42	Iodent[c] (59%), Long Ear (20%), Minnesota 13[c] (13%), Troyer Reid[c] (5%)
PHMK0	BSSS[b] C0 (38%), Southern U.S. Landrace Synthetic (21%), BSSS[b] (13%), Dockendorf 101[c] (13%)
PHMM9	BSSS[b] C0 (53%), Dockendorf 101[c] (25%), Maiz Amargo[c] (13%)
PHN46	Southern U.S. Landrace Synthetic (12%), Iodent[c] (10%), Lancaster Low Breakage (9%), Osterland Yellow Dent[c] (9%), Funks G4949 (8%), Minnesota 13[c] (6%), Midland Yellow Dent[c] (6%)
PHN65	BSSS[b] (50%), Minesota 13[c] (6%), Osterland Yellow Dent[c] (6%), SRS 303[c] (6%), Iodent[c] (6%), Reid[c] (6%)
PHP38	BSSS[b] C0 (66%), Maiz Amargo[c] (13%), BSSS[b] (13%)
PHP85	BSSS[b] C0 (48%), BSSS[b] (38%), Maiz Amargo[c] (6%)
PHPE5	Iodent[c] (22%), Southern U.S. Landrace Synthetic (9%), Midland Yellow Dent[c] (9%), Minnesota 13[c] (8%), Long Ear (8%), Coker 616 (6%), Funks G4949 (6%), Illinois Long Ear (5%), Illinois Two Ear (5%)
PHR03	Iodent[c] (25%), Minnesota 13[c] (11%), Long Ear (8%), Southern U.S. Landrace Synthetic (6%), Midland Yellow Dent[c] (6%), Lancaster Sure Crop[c] (6%)
PHR63	Iodent[c] (29%), Coker 616 (13%), Minnesota 13[c] (10%), Long Ear (10%), Lancaster Sure Crop[c] (6%), Midland Yellow Dent[c] (6%), Southern U.S. Landrace Synthetic (5%)
PHR92	BSSS[b] C0 (69%), Maiz Amargo[c] (25%)
PHT11	BSSS[b] C0 (47%), BSSS[b] (25%), Maiz Amargo[c] (13%), Alberta Flint (6%), Osterland Yellow Dent[c] (6%)
PHT55	BSSS[b] C0 (69%), Maiz Amargo[c] (25%)
PHV25	Iodent[c] (30%), Midland Yellow Dent[c] (13%), Long Ear (10%), Southern U.S. Landrace Synthetic (9%), Minnesota 13[c] (7%), Funks G4949 (6%), Illinois Long Ear (6%), Illinois Two ear (6%)

Table 1 Continued

A632	Pedigree background[a]
PHV35	BSSS[b] (50%), BSSS[b] C0 (34%), Maiz Amargo[c] (13%)
PHV78	Iodent[c] (15%), Southern U.S. Landrace Synthetic (14%), Midland Yellow Dent[c] (13%), Funks G4949 (9%), Illinois Long Ear (6%), Illinois Two Ear (6%), Lancaster Low Breakage (6%), Long Ear (5%), Minnesota 13[e] (5%), Tuson B[d] (5%)
PHV94	BSSS[b] C0 (53%), Dockendorf 101[c] (25%), Maiz Amargo[c] (13%)
PHW52	BSSS[b] (50%), BSSS[b] C0 (34%), Maiz Amargo[c] (13%)
PHW53	Iodent[c] (21%), Osterland Yellow Dent[c] (11%), Minnesota 13[e] (10%), Long Ear (7%), Lancaster Low Breakage (6%), SRS 303[f] (6%), Reid[c] (6%), Southern U.S. Landrace Synthetic (5%)
PHWK9	Maiz Amargo[c] (50%), BSSS[b] C0 (50%)
PHZ38	BSSS[b] (50%), BSSS[b] C0 (41%)
PHZ51	Osterland Yellow Dent[c] (14%), Lancaster Low Breakage (13%), Southern U.S. Landrace Synthetic (9%), Minnesota 13[e] (8%), Funks G4949 (6%), SRS 303[f] (6%), Iodent[c] (6%), Reid[c] (6%)

[a] Contributions of 5% or greater by pedigree are provided
[b] Iowa Stiff Stalk Synthetic
[c] Open-pollinated variety
[d] Derived from Tuson, an open-pollinated variety from the West Indies
[e] Population derived from Minnesota 13 open-pollinated variety
[f] Stiff Root and Stalk or Stalk Rot Synthetic selection from Krug
[g] Dawes open-pollinated variety from Nebraska most likely from Reid obtained from Mount Haleb, Wisconsin
[h] Smith top-cross derived from HAT0 fling synthetic
[i] Northwest Dent, open-pollinated variety once grown in northwest and north central U.S.
[j] Synthetic from Mississippi
[k] Composite of Southern U.S. prolific germplasm and Corn Belt lines made by W. L. Brown in the 1960's; known as "BS11" at Iowa State University
[l] Hybrid once sold by Dockendorf

Materials and methods

DNA was extracted from 58 maize inbred lines (Table 1) and from four maize hybrids (Pioneer hybrids 3183, 3377, 3732, and 3747). The 58 inbreds encompass a broad range of genetic diversity for Corn Belt materials, including pairs of lines that span pedigree relationships from unrelated to highly related. Among these inbred lines were 13 sets of triplets (a progeny line and both its parents) that provided opportunities for tests of inheritance and/or reliable band scoring. In addition, four hybrids were also profiled, providing additional opportunities to check the scoring and inheritance of polymorphisms. Initial DNA extractions were made using the CTAB procedure (Saghai-Maroof et al. 1984). Subsequent DNA extractions were performed using a proprietary method for which patent protection is being sought. Both methods provide DNA suitable for amplification by these SSRs and gave equivalent results. SSR loci were individually amplified using DNA of each inbred and hybrid using protocols described by Chin et al. (1996), except that fluorescent-labeled primers were used. Samples containing 0.5 µl of the PCR products, 0.5 µl of GENESCAN 500 internal lane standard labeled with N, N, N', N'-tetramethyl-6-carboxyrhodamine (TAMRA) (Perkin Elmer-Applied Biosystems), and 50% formamide were heated at 92°C for 2 min, placed on ice, then loaded on 6% denaturing acrylamide gels. DNA samples were electrophoresed (29 W) for 7 h on an ABI Model 373A automative DNA sequencer/fragment analyzer equipped with GENESCAN 672 software v. 1.2 (Perkin Elmer-Applied Biosystems). DNA fragments were sized automatically using the "local Southern" sizing algorithm (Elder and Southern 1987). PCR products from individual samples were assigned to specific alleles at each locus based on "binning" of a range of sizes (±0.5 bp) as determined by ABI GeneScan™ and GENOTYPER™ software using the "local Southern" algorithm. Primer pairs for 200 potentially useful SSR loci were identified from the sequence data of maize that were published in Genbank, from di-repeat libraries made by Ben Burr (Brookhaven National Laboratory) and Lynn Senior (North Carolina State University), and from additional sequences available within Pioneer Hi-Bred International, Inc. An initial screen of nine inbred lines was used to evaluate utility (Chin et al. 1996). Sequence data for primers to amplify these SSRs are available via the electronic maize database (Maize DB, Polacco 1996). Attempts were made to profile all of the 58 inbred lines and four hybrids with these SSRs. It was possible to obtain profiles for all of the inbreds and hybrids included in this survey for 131 SSRs (see Table 2). Genomic locations for most SSRs are provided according to the nomenclature used in Coe (1996). Among this set of SSRs, 59 (45%) were di-repeats, 36 (27%) were tri-repeats, 21 (16%) were tetra-repeats, 7 (5%) were penta-repeats, 5 (4%) were hexa-repeats, 2 (2%) were septa-repeats, and 1 (1%) was an octa-repeat.

RFLP data were obtained by Linkage Genetics (Salt Lake City, Utah) using DNA extraction and other protocols described by Helentjaris et al. (1985). Eighty single-locus probes that collectively sampled every chromosome arm were used.

PIC values were calculated using the algorithm:

$$PIC = 1 - \sum^{n} f_i^2 \quad i - 1,$$

where f_i^2 is the frequency of the i^{th} allele.

PIC provides an estimate of the discriminatory power of a locus by taking into account, not only the number of alleles that are expressed, but also the relative frequencies of those alleles. PIC values range from 0 (monomorphic) to 1 (very highly discriminative, with many alleles in equal frequencies). For example, a marker locus that reveals five alleles, but where one allele is found in very high frequency (e.g., freq. = 0.9), has overall less discriminatory capability than a locus that also has five alleles, but in which those alleles are found in more equal frequencies.

Genetic distances between pairs of inbred lines from SSR and RFLP data were calculated from comparisons of the band scores using a modified Nei's distance (Nei and Li 1979). Pedigree distances between pairs of inbreds were calculated from 1-Malecot's Coefficient of relatedness (Malecot 1948). Associations among inbreds from SSR, RFLP and pedigree data were revealed using average linkage cluster analysis.

Results

SSRs that failed to amplify against the majority of inbreds or which gave amplified products that could not be clearly resolved were re-amplified and electrophoresed a second time. If results were still poor, then primers were re-designed (designated with ' – 2' following the SSR locus name) for further evaluation. If amplified products still failed to yield clearly scorable profiles for less than 95% of the inbred lines, then those SSRs were discarded from this study. This exercise resulted in scorable data being obtained for the 58 inbreds and four hybrids from 131 SSRs (Table 2). Primers with different sequences for loci already published (Coe 1996) may result in amplification products with different molecular weights from those obtained using the initial primer sequences.

Thirteen parent-progeny triplets were available for the examination of inheritance and scoring accuracy. For SSRs, non-Mendelian scores (where an amplified product was scored in a progeny inbred that had not been scored in one or both parental inbreds) ranged from 0 to 7 of the SSRs (0–5.3% of SSRs) per triplet. The mean was 2.85 incidences of non-Mendelian scoring (2.2% of all SSRs) per triplet. For RFLPs the range of non-Mendelian scores was from 0 to 7 RFLPs per triplet (0–8.8% of RFLPs per triplet). The mean for RFLPs was 2.85 (3.6% of RFLPs) incidences of non-Mendelian scoring per triplet.

Twenty five of the 131 SSRs were associated with one or more incidences of non-Mendelian scoring in the triplets. One SSR (bngl 619), a di-repeat, was so detected in four triplets; phi 011, a tri-repeat resulted in non-Mendelian scores for three triplets; six SSRs gave rise to non-Mendelian scores in each of two triplets; the remaining 17 SSRs that gave rise to non-Mendelian scores did so in only single triplets. Of all the SSRs implicated in non-Mendelian scoring, ten were di-repeats (16% of all di-repeats), eight were tri-repeats (24% of all tri-repeats), five were tetra-repeats (24% of all tetra-repeats), and two penta-repeats (33% of all penta-repeats).

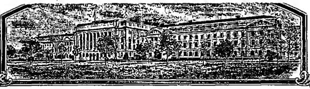
Incidences of non-Mendelian scoring (absence of a parental band in a hybrid or presence of a non-parental band in a hybrid) expressed as a percentage of the 131 SSR loci for each hybrid were 3% for Pioneer brand hybrids 3183 and 3377 and 1.5% for Pioneer brand hybrids 3732 and 3747. The mean was 2.3% per triplet. Of the 12 instances of non-Mendelian scoring that were found, 11 were due to the absence of one of the inbred parental bands in the hybrid and one resulted from the presence of a band in the hybrid that was scored in neither parent.

PIC values for SSRs are presented in Table 3. PIC values for SSRs ranged from 0.06 to 0.91; the mean PIC for SSRs was 0.62. Summary data for numbers of bands and PIC values for each repeat class are presented in Table 4. Di-repeats gave high PIC values (0.70). Other frequently used classes (tri- and tetra-repeats) resulted in PIC values of 0.53 and 0.59, respectively.

Associations among inbreds on the basis of pedigree, RFLP and SSR data are presented in Figs. 1, 2 and 3, respectively. Associations of inbreds on the basis of pedigree (Fig. 1) were similar to that which could be expected on the basis of either marker method (Figs. 2 and 3). Very similar associations of inbreds were revealed from analyses of the RFLP and the SSR data (Figs. 2 and 3). The correlations of pairwise distances

Table 2 a SSR markers and map locations; primer sequences are given by Coe (1996)

SSR Locus	Genomic Location	SSR Locus	Genomic Location
phi056	1.01	bngl249	6.01
phi097	1.01	bngl107	6.02
bngl182	1.03	bngl480	6.03
bngl439	1.03	phi031	6.03
phi001	1.04	bngl176	6.04
bngl421	1.05	phi070	6.06
bngl615	1.07	phi025	6.07
bngl100	1.08	phi078	6.07
phi011	1.10	phi057	7.01
phi055	1.10	phi112	7.01
phi094	1.10	phi114	7.02
bngl504	1.11	bngl657	7.03
phi064	1.11	bngl434	7.03
bngl108	2.04	bngl155	7.04
bngl166	2.04	phi082	7.06
bngl420	2.04	bngl669	8.03
phi083	2.04	phi115	8.03
bngl602	3.04	phi119	8.03
nc030	3.04	bngl240	8.04
phi029	3.04	phi014	8.05
phi073	3.05	phi060	8.05
bngl197	3.07	phi015	8.08
phi072	4.01	phi080	8.08
phi021	4.02	phi017	9.02
bngl490	4.04	phi028	9.02
bngl667	4.04	phi033	9.02
bngl252	4.05	phi044	9.02
phi096	4.05	bngl127	9.03
phi092	4.08	bngl244	9.03
phi093	4.08	bngl430	9.03
bngl589	4.10	phi022	9.03
phi006	4.10	phi027	9.03
phi019	4.10	phi061	9.03
phi076	4.10	phi065	9.03
phi024	5.00	phi016	9.04
bngl143	5.01	phi042	9.04
bngl105	5.02	bngl128	9.07
phi113	5.02	bngl619	9.07
phi008	5.03	phi059	10.02
bngl653	5.04	phi063	10.02
bngl278	5.06	bngl640	10.03
bngl609	5.06	phi071	10.04
phi085	5.06	phi084	10.04
bngl386	5.09	bngl236	10.06
bngl238	6.00	bngl594	10.06
phi075	6.00		

No.  200200187

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

Pioneer Hi-Bred International, Inc.

Whereas, THERE HAS BEEN PRESENTED TO THE

Secretary of Agriculture

AN APPLICATION REQUESTING A CERTIFICATE OF PROTECTION FOR AN ALLEGED DISTINCT VARIETY OF SEXUALLY REPRODUCED, OR TUBER PROPAGATED PLANT, THE NAME AND DESCRIPTION OF WHICH ARE CONTAINED IN THE APPLICATION AND EXHIBITS, A COPY OF WHICH IS HEREUNTO ANNEXED AND MADE A PART HEREOF, AND THE VARIOUS REQUIREMENTS OF LAW IN SUCH CASES MADE AND PROVIDED HAVE BEEN COMPLIED WITH, AND THE TITLE THERETO IS, FROM THE RECORDS OF THE PLANT VARIETY PROTECTION OFFICE, IN THE APPLICANT(S) INDICATED IN THE SAID COPY, AND WHEREAS, UPON DUE EXAMINATION MADE, THE SAID APPLICANT(S) IS (ARE) ADJUDGED TO BE ENTITLED TO A CERTIFICATE OF PLANT VARIETY PROTECTION UNDER THE LAW.

NOW, THEREFORE, THIS CERTIFICATE OF PLANT VARIETY PROTECTION IS TO GRANT UNTO THE SAID APPLICANT(S) AND THE SUCCESSORS, HEIRS OR ASSIGNS OF THE SAID APPLICANT(S) FOR THE TERM OF TWENTY YEARS FROM THE DATE OF THIS GRANT, SUBJECT TO THE PAYMENT OF THE REQUIRED FEES AND PERIODIC REPLENISHMENT OF VIABLE BASIC SEED OF THE VARIETY IN A PUBLIC REPOSITORY AS PROVIDED BY LAW, THE RIGHT TO EXCLUDE OTHERS FROM SELLING THE VARIETY, OR OFFERING IT FOR SALE, OR REPRODUCING IT, OR IMPORTING IT, OR EXPORTING IT, R CONDITIONING IT FOR PROPAGATION, OR STOCKING IT FOR ANY OF THE ABOVE PURPOSE, OR CONDITIONING IT PROPAGATION, OR STOCKING IT FOR ANY OF THE ABOVE PURPOSE, OR USING IT IN PRODUCING A HYBRID OR RENT VARIETY THEREFROM, TO THE EXTENT PROVIDED BY THE PLANT VARIETY PROTECTION ACT. (84 2, AS AMENDED, 7 U.S.C. 2321 ET SEQ.)

CORN, FIELD

'PH8CW'

In Testimony Whereof, *I have hereunto set my hand and caused the seal of the* Plant Variety Protection Office *to be affixed at the City of Washington, D.C. this first day of July, in the year two thousand and four.*

Attest:

Commissioner
Plant Variety Protection Office
Agricultural Marketing Service

Agriculture

APPENDIX 5

REPRODUCE LOCALLY. *Include form number and date on all reproductions.* FORM APPROVED - OMB NO. 0581-0055

U.S. DEPARTMENT OF AGRICULTURE
AGRICULTURAL MARKETING SERVICE
SCIENCE AND TECHNOLOGY DIVISION - PLANT VARIETY PROTECTION OFFICE

APPLICATION FOR PLANT VARIETY PROTECTION CERTIFICATE
(Instructions and information collection burden statement on reverse)

The following statements are made in accordance with the Privacy Act of 1974 (5 U.S.C. 552a) and the Paperwork Reduction Act (PRA) of 1995.

Application is required in order to determine if a plant variety protection certificate is to be issued (7 U.S.C. 2421). Information is held confidential until certificate is issued (7 U.S.C. 2426).

1. NAME OF OWNER
Pioneer Hi-Bred International, Inc.

2. TEMPORARY DESIGNATION OR EXPERIMENTAL NUMBER

3. VARIETY NAME
PH8CW

4. ADDRESS (Street and No. or RFD No., City, State and Zip Code, and Country)
7301 NW 62nd Avenue
P.O. Box 85
Johnston, IA 50131-0085

5. TELEPHONE (include area code)
515/270-4051

6. FAX (include area code)
515/253-2125

FOR OFFICIAL USE ONLY
PVPO NUMBER
200200187

FILING DATE
June 6, 2002

7. IF THE OWNER NAMED IS NOT A "PERSON", GIVE FORM OF ORGANIZATION (corporation, partnership, association, etc.)
Corporation

8. IF INCORPORATED, GIVE STATE OF INCORPORATION)
IOWA

9. DATE OF INCORPORATON
March 5, 1999

10. NAME AND ADDRESS OF OWNER REPRESENTATIVE(S) TO SERVE IN THIS APPLICATION (FIRST PERSON LISTED WILL RECEIVE ALL PAPERS)
Steven R. Anderson
Research and Product Development
P.O. Box 85
Johnston, IA 50131-0085

FEES RECEIVED
FILING & EXAMINATION FEES:
$ 2705.00
DATE 6/6/02
CERTIFICATION FEE:
$ 432.00
DATE 6/18/04

11. TELEPHONE (include area code)
515/270-4051

12. FAX (include area code)
515/253-2125

13. E_MAIL
Steven.Anderson@Pioneer.com

14. CROP KIND NAME (Common name)
CORN

15 GENUS AND SPECIES NAME OF CROP
Zea Mays

16. FAMILY NAME (Botanical)
Gramineae

17. IS THE VARIETY A FIRST GENERATION HYBRID?
☐ Yes ☒ No

18. CHECK APPROPRIATE BOX FOR EACH ATTACHMENT SUBMITTED *(Follow instructions on reverse)*
a. ☒ Exhibit A. Origin and Breeding History of the Variety
b. ☒ Exhibit B. Statement of Distinctness
c. ☒ Exhibit C. Objective Description of the Variety
d. ☐ Exhibit D. Additional Description of the Variety *(Optional)*
e. ☒ Exhibit E. Statement of the Basis of the Owner's Ownership
f. ☒ Voucher Sample (2500 viable untreated seeds or, for tuber propagated varieties verification that tissue culture will be deposited and maintained in an approved public repository)
g. ☒ Filing and Examination Fee ($2,705), made payable to "Treasurer of the United States" *(Mail to Plant Variety Protection Office))*

19. DOES THE OWNER SPECIFY THAT SEED OF THIS VARIETY BE SOLD AS A CLASS OF CERTIFIED SEED? See Section 83(a) of the Plant Variety Protection Act)
☐ YES (If "yes", answer items 20 and 21 below) ☒ NO (If "no", go to item 22)

20. DOES THE OWNER SPECIFY THAT SEED OF THIS VARIETY BE LIMITED AS TO NUMBER OF CLASSES? ☐ YES ☐ NO
IF "YES", WHICH CLASSES? ☐ FOUNDATION ☐ REGISTERED ☐ CERTIFIED

21. DOES THE OWNER SPECIFY THAT SEED OF THIS VARIETY BE LIMITED AS TO NUMBER OF GENERATIONS? ☐ YES ☐ NO
IF "YES" SPECIFY THE? ☐ FOUNDATION ☐ REGISTERED ☐ CERTIFIED
Number 1,2,3 etc.
(If additional explanation is necessary, please use the space indicated on the reverse.)

22. HAS THE VARIETY (INCLUDING ANY HARVESTED MATERIAL) OR A HYBRID PRODUCED FROM THIS VARIETY BEEN SOLD, DISPOSED OF, TRANSFERRED, OR USED IN THE U.S. OR OTHER COUNTRIES?
☒ YES ☐ NO
IF YES, YOU MUST PROVIDE THE DATE OF FIRST SALE, DISPOSITION, TRANSFER, OR USE FOR EACH COUNTRY AND THE CIRCUMSTANCES. *(Please use space indicated on reverse)*

23. IS THE VARIETY OR ANY COMPONENT OF THE VARIETY PROTECTED BY INTELLECTUAL PROPERTY RIGHT (PLANT BREEDER'S RIGHT OR PATENT)?
☐ YES ☒ NO
IF YES, PLEASE GIVE COUNTRY, DATE OF FILING OR ISSUANCE AND ASSIGNED REFERENCE NUMBER. *(Please use space indicated on reverse.)*

24. The owner(s) declare that a viable sample of basic seed of the variety will be furnished with application and will be replenished upon request in accordance with such regulations as may be applicable, or for a tuber propagated variety a tissue culture will be deposited in a public repository and maintained for the duration of the certificate.

The undersigned owner(s) is(are) the owner of this sexually reproduced or tuber propagated plant variety, and believe(s) that the variety is new, distinct, uniform, and stable as required in Section 42, and is entitled to protection under the provisions of Section 42 of the Plant Variety Protection Act.

Owner(s) is(are) informed that false representation herein can jeopardize protection and results in penalties.

SIGNATURE OF OWNER		SIGNATURE OF OWNER	
		Steven R Anderson	
NAME (Please print or type)		NAME (Please print or type) Steven R. Anderson	
CAPACITY OR TITLE	DATE	CAPACITY OR TITLE Research Scientist	DATE 5-15-02

S&T-470 (04-01)designed by the Plant Variety Protection Office with WordPerfect 6.0a. Replaces STD-470 (02-99) which is obsolete. *(See reverse for instructions and information collection burden statement)*

INSTRUCTIONS

200200187

GENERAL: To be effectively filed with the Plant Variety protection Office (PVPO), ALL of the following items must be received in the PVPO: (1) Completed application form signed by the owner; (2) completed Exhibits A, B, C, E; (3) for a seed reproduced variety at least 2,500 viable untreated seeds, for a hybrid variety at least 2,500 untreated seeds of each line necessary to reproduce the variety, or for tuber reproduced varieties verification that a viable *(in the sense that it will reproduce an entire plant)* tissue culture will be deposited and maintained in a approved public repository; (4) check drawn on a U.S. bank for $2705 ($320 filing fee and $2,385 examination fee), payable to "Treasurer of the United States" *(See Section 97.6 of the Regulations and Rules of Practice.)* Partial applications will be held in the PVPO for not more than 90 days, then returned to the applicant as unfiled. Mail application and other requirements to Plant Variety Protection Office, AMS, USDA, Room 400, NAL Building, 10301 Baltimore Avenue, Beltsville, MD 20705-2351. Retain one copy for your files. All items on the face of the application are self explanatory unless noted below. Corrections on the application form and exhibits must be initialed and dated. DO NOT use masking materials to make corrections. If a certificate is allowed, you will be requested to send a check payable to "Treasurer of the United States" in the amount of $320 for issuance of the certificate. Certificates will be issued to owner, not licensee or agent.

Plant Variety Protection Office
Telephone: (301)504-5518
FAX: (301)504-5291
Homepage: http://www.ams.usda.gov/science/pvp.htm

ITEM

18a. Give: (1) the genealogy, including public and commercial varieties, lines, or clones used, and the breeding method;
(2) the details of subsequent stages of selection and multiplication;
(3) evidence of uniformity and stability; and
(4) the type and frequency of variants during reproduction and multiplication and state how these variants may be identified.

18b. Give a summary of the variety's distinctness. Clearly state how this application variety may be distinguished from all other varieties in the same crop. If the new variety is most similar to one variety or a group of related varieties:
(1) identify these varieties and state all differences objectively;
(2) attach statistical data for characters expressed numerically and demonstrate that these are clear differences; and
(3) submit, if helpful, seed and plant specimens of photographs (prints) of seed and plant comparisons which clearly indicate distinctness.

18c. Exhibit C forms are available from the PVPO for most crops; specify crop kind. Fill in Exhibit C (Objective Description of Variety) form as completely as possible to describe your variety.

18d. Optional additional characteristics and/or photographs. Describe any additional characteristics that cannot be accurately conveyed in Exhibit C. Use comparative varieties as is necessary to reveal more accurately the characteristics that are difficult to describe, such as plant habit, plant disease resistance, etc.

18e. Section 52(5) of the Act required applicants to furnish a statement of the basis of the applicant's ownership. An Exhibit E form is available from the PVPO.

19. If "Yes" is specified *(seed of this variety be sold by variety name only, as a class of* certified seed), the applicant MAY NOT reverse this affirmative decision after the variety has been sold and so labeled, the decision published, or the certificate issued. However, if "No" has been specified, applicant may change the choice. *(See Regulations and Rules of Practice, Section 7.103).*

22. *See Sections 41, 42, and 43 of the Act and Section 97.5 of the regulations for eligibility requirements.*

23. See Section 5.5 of the Act for instructions on claiming the benefit of an earlier filing date

21. CONTINUED FROM FRONT *(Please provide a statement as to the limitation and sequence of generations that may be certified.)*

22. CONTINUED FROM FRONT (Please provide the date of first sale, disposition, transfer, or use for each country and the circumstances, if the variety (including any harvested material) or a hybrid produced from this variety ahs been sold, disposed of, transferred, or used in the U>S> or other countries.)

Nov. 1, 2001 United States, Italy, Canada

23. CONTINUED FROM FRONT *(Please give the country, date of filing or issuance, and assigned reference number, if the variety or any component of the variety is protected by intellectual property right (Plant Breeder's Right or Patent).*

NOTES; It is the responsibility of the applicant/owner to keep the PVPO informed of any changes of address or change of ownership or assignment or owner's representative during the life of the application/certificate. There is no charge for filing a change of address. The fee for filing a change of ownership or assignment or any modification of owner's name is specified in Section 97.175 of the regulations. *(See Section 101 of the Act, and Sections 97.130, 97.131, 97.175(h) of Regulations and Rules of Practice.)*

To avoid conflict with other variety names in use, the applicant should check the variety names proposed by contacting: Seed Branch, AMS, USDA, Room 213, Building 306, Beltsville Agricultural Research Center--East, Beltsville, MD 20705. Telephone: (301) 504-8089. http://www.ams.usda.agov/lsg/seed/ls-sd.htm

Exhibit A. Origin and Breeding History

200200187

Pedigree: PH24E/PH1CA)XA4K12K1X

Pioneer Line PH8CW, Zea mays L., a dent corn inbred, was developed by Pioneer Hi-Bred International, Inc. from the single cross hybrid PH24E (Certificate No. 9600204) X PH1CA (PVP Certificate No. 9800386) using the pedigree method of plant breeding. Varieties PH24E and PH1CA are proprietary inbred lines of Pioneer Hi-Bred International, Inc. Selfing was practiced from the above hybrid for seven generations using pedigree selection. During line development, crosses were made to inbred testers for the purpose of estimating the line's combining ability. Yield trials were grown at Windfall, IN as well as other Pioneer research locations. After initial testing, additional hybrid combinations have been evaluated and subsequent generations of the line have been grown and hand-pollinated with observations again made for uniformity.

Variety PH8CW has shown uniformity and stability for all traits as described in Exhibit C - "Objective Description of Variety". It has been self-pollinated and ear-rowed five generations with careful attention paid to selection criteria and uniformity of plant type to assure genetic homozygousity and phenotypic stability. The line has been increased both by hand and in isolated fields with continued observations for uniformity and stability, and for 3 generations during the final stages of inbred development and seed multiplication. Very high standards for genetic purity have been established morphologically using field observations and electrophoretically using sound lab molecular marker methodology.

No variant traits have been observed or are expected in PH8CW.

The criteria used in the selection of PH8CW were yield, both per se and in hybrid combinations; late season plant health, grain quality, stalk lodging resistance, and kernel size, especially important in production. Other selection criteria include: ability to germinate in adverse conditions; disease and insect resistance; pollen yield and tassel size.

Exhibit A: Developmental history for PH8CW

Season/Year Pedigree Grown	Inbreeding Level of Pedigree Grown
May/1995 PH24E	F0
May/1995 PH1CA	F0
Nov/1995 PH24E/PH1CA	F1
May/1996 PH24E/PH1CA)X	F2
May/1997 PH24E/PH1CA)XA4	F3
Nov/1997 PH24E/PH1CA)XA4	F3
May/1998 PH24E/PH1CA)XA4K1	F4
Nov/1998 PH24E/PH1CA)XA4K12	F5
May/1999 PH24E/PH1CA)XA4K12K1	F6
Oct/1999 PH24E/PH1CA)XA4K12K1X	F7

*PH8CW was selfed and ear-rowed from F2 through F6 generation.
#Uniformity and stability were established from F4 through F6 generation and beyond when seed supplies were increased.

Exhibit B: Novelty Statement

Variety PH8CW mostly resembles Pioneer Hi-Bred International, Inc. proprietary inbred line PH24E (PVP Certificate No. 9600204). Figures 1 & 2 demonstrate cob color differences between the two varieties. Tables 1A and 1B show two sample t-tests on data collected primarily in Johnston and Dallas Center, IA. The traits collectively show measurable differences between the two varieties.

Variety PH8CW has a narrower leaf angle (18.6 degrees vs 25.1degrees) than variety PH24E (Table 1A, 1B).

Variety PH8CW has a greater husk length (21.5cm vs 17.9cm) than variety PH24E (Table 1A, 1B).

Variety PH8CW has a pink cob (2.5YR 7/6) vs. a red cob color (2.5YR 5/6) for PH24E (Figure 1 &2).

Figure 1. PH8CW

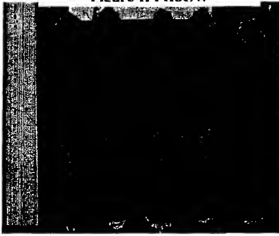


Figure 2. PH24E

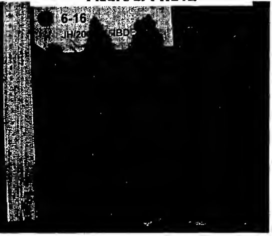


Exhibit B: Novelty Statement Tables

Table 1A: Data from Johnston and Dallas Center, IA broken out by 3 different locations in 2001 are supporting evidence for differences between PH8CW and PH24E. Locations had different environmental conditions. Environments had different planting dates and were in different fields. A two-sample t-test was used to compare differences between means.

TRAIT	year	station	Variety_1	Variety_2	Count_1	Count_2	Mean_1	Mean_2	Mean_Diff	StdDeviation_1	StdDeviation_2	StdError_1	StdError_2	DF_Pooled	t-Value_Pooled	Prob_(2-tail)_Pooled
husk length (cm)	2001	AD	PH8CW	PH24E	5	5	21.4	17.8	3.6	0.894	0.837	0.400	0.374	8	6.6	0.000
husk length (cm)	2001	DC	PH8CW	PH24E	5	5	21.2	17.6	3.6	0.447	0.894	0.200	0.400	8	8.0	0.000
husk length (cm)	2001	JH	PH8CW	PH24E	5	5	21.8	18.2	3.6	1.304	0.837	0.583	0.374	8	5.2	0.001
leaf angle (degrees)	2001	AD	PH8CW	PH24E	5	5	20.2	28.6	-8.4	5.718	3.715	2.557	1.661	8	-2.8	0.025
leaf angle (degrees)	2001	DC	PH8CW	PH24E	5	5	16.6	23.2	-6.6	3.782	4.382	1.691	1.960	8	-2.5	0.034
leaf angle (degrees)	2001	JH	PH8CW	PH24E	5	5	19.0	23.6	-4.6	1.732	3.050	0.775	1.364	8	-2.9	0.019

20020018 7

6

Exhibit B. Novelty Statement Tables

Table 1B: Summary data from Johnston and Dallas Center, IA across environments in 2001 are supporting evidence for differences between PH8CW and PH24E. Environments had different planting dates and were in different fields. A two-sample t-test was used to compare differences between means.

TRAIT	year	variety-1	variety-2	Count-1	Count-2	Mean-1	Mean-2	Mean_Diff	StdDeviation-1	StdDeviation-2	StdError-1	StdError-2	DF-Pooled	t-Value_Pooled	Prob_(2-tail)_Pooled
leaf angle (degrees)	2001	PH8CW	PH24E	15	15	18.6	25.1	-6.5	4.085	4.307	1.055	1.112	28	-4.3	0.000
husk length (cm)	2001	PH8CW	PH24E	15	15	21.5	17.9	3.6	0.915	0.834	0.236	0.215	28	11.3	0.000

200200187

United States Department of Agriculture, Agricultural Marketing Service
Science Division, Plant Variety Protection Office
National Agricultural Library Building, Room 500
Beltsville, MD 20705

Objective Description of Variety
Corn (Zea mays L.)

Name of Applicant (s)	Variety Seed Source	Variety Name or Temporary Designation
Pioneer Hi-Bred International, Inc.		PH8CW
Address (Street & No., or RFD No., City, State, Zip Code and Country		FOR OFFICIAL USE
7301 NW 62nd Avenue, P.O. Box 85, Johnston, Iowa 50131-0085		PVPO Number **200200187**

Place the appropriate number that describes the varietal characters typical of this inbred variety in the spaces below. Right justify whole numbers by adding Leading zeroes if necessary. Completeness should be striven for to establish an adequate variety description. Traits designated by an '*' are considered Necessary for an adequate variety description and must be completed.

COLOR CHOICES (Use in conjunction with Munsell color code to describe all color choices: describe #25 and #26 in Comments section):

01=Light Green	06=Pale Yellow	11=Pink	16=Pale Purple	21=Buff
02=Medium Green	07=Yellow	12=Light Red	17=Purple	22=Tan
03=Dark Green	08=Yellow Orange	13=Cherry Red	18=Colorless	23=Brown
04=Very Dark Green	09=Salmon	14=Red	19=White	24=Bronze
05=Green-Yellow	10=Pink-Orange	15=Red & White	20=White Capped	25=Variegated (Describe)
				26=Other (Describe)

STANDARD INBRED CHOICES
(Use the most similar (in background and maturity) of these to make comparisons based on grow-out trial data):

Yellow Dent Families:		Yellow Dent (Unrelated):	Sweet Corn:
Family	Members	Co109, ND246,	C13, Iowa5125, P39, 2132
B14	CM105, A632, B64, B68	Oh7, T232,	
B37	B37, B76, H84	W117, W153R,	Popcorn:
B73	N192, A679, B73, NC268	W18BN	SG1533, 4722, HP301, HP7211
C103	Mo17, Va102, Va35, A682		
Oh43	A619, MS71, H99, Va26	White Dent:	Pipecorn:
WF9	W64A, A554, A654, Pa91	C166, H105, Ky228	Mo15W, Mo16W, Mo24W

Groups on Lynx/Osborn/Grunst/98-99PVP

EXHIBIT C: PH8CW

Application Variety Data				Standard Variety Data		
1. TYPE: (describe Intermediate types in Comments section):				Standard Variety Name		
2	1=Sweet 2=Dent 3=Flint 4=Flour 5=Pop 6=Ornamental Dent			MO17		
2. REGION WHERE DEVELOPED IN THE U.S.A.:				Standard Seed Source		
5	1=Northwest 2=Northcentral 3=Northeast 4=Southeast 5=Southcentral 6=Southwest 7=Other			PI 558532		

3. MATURITY (In Region of Best Adaptability; show Heat Unit formula in 'Comments' section)

DAYS	HEAT UNITS		DAYS	HEAT UNITS
068	1,358.0	From emergence to 50% of plants in silk	074	1,559.3
068	1,359.3	From emergence to 50% of plants in pollen	069	1,404.7
002	0,046.0	From 10% to 90% pollen shed	002	0,066.0
		From 50% silk to optimum edible quality		
		From 50% silk to harvest at 25% moisture		

4. PLANT:

		Standard Deviation	Sample Size		Standard Deviation	Sample Size
204.3	cm Plant Height (to tassel tip)	09.81	03	215.3	06.43	03
075.3	cm Ear Height (to base of top ear node)	10.26	03	078.0	03.61	03
014.6	cm Length of Top Ear Internode	01.39	03	014.9	00.95	03
0.0	Average Number of Tillers/plant	00.01	03	0.0	00.01	03
0.9	Average Number of Ears per Stalk	00.05	03	0.9	00.03	03
3	Anthocyanin of Brace Roots: 1=Absent 2=Faint 3=Moderate 4=Dark 5=Very Dark			1		

5. LEAF:

		Standard Deviation	Sample Size		Standard Deviation	Sample Size
08.7	cm Width of Ear Node Leaf	01.70	03	09.6	00.35	03
83.1	cm Length of Ear Node Leaf	03.95	03	70.4	03.99	03
06	Number of leaves above top ear	00.31	03	06	00.12	03
19	Degrees Leaf Angle (measure from 2nd leaf above ear at anthesis to stalk above leaf)	01.83	03	26	06.36	03
03	Leaf Color (Munsell code) 7.5GY34			03	5GY34	
1	Leaf Sheath Pubescence (Rate on scale from 1=none to 9=like peach fuzz)			2		
	Marginal Waves (Rate on scale from 1=none to 9=many)					
	Longitudinal Creases (Rate on scale from 1=none to 9=many)					

6. TASSEL:

		Standard Deviation	Sample Size		Standard Deviation	Sample Size
09	Number of Primary Lateral Branches	02.50	03	06	00.31	03
31	Branch Angle from Central Spike	07.23	03	36	03.47	03
57.5	cm Tassel Length (from top leaf collar node to tassel tip)	00.31	03	63.2	02.08	03
6	Pollen Shed (rate on scale from 0=male sterile to 9=heavy shed)			6		
14	Anther Color (Munsell code) 5R46			01	2.5GY88	
17	Glume Color (Munsell code) 10RP26			01	5GY58	
2	Bar Glumes (Glume Bands): 1=Absent 2=Present			1		

Application Variety Data PH8CW Page 2 Standard Variety Data

	Application Variety Data			Standard Variety Data	
7a. EAR (Unhusked Data):					
11	Silk Color (3 days after emergence) (Munsell code)	10RP48		01	2.5GY86
03	Fresh Husk Color (25 days after 50% silking) (Munsell code)	5GY56		02	5GY68
21	Dry Husk Color (65 days after 50% silking) (Munsell code)	2.5Y92		21	2.5Y8.54
2	Position of Ear at Dry Husk Stage: 1= Upright 2= Horizontal 3= Pendant			1	
4	Husk Tightness (Rate of Scale from 1=very loose to 9=very tight)			3	
2	Husk Extension (at harvest): 1=Short (ears exposed) 2=Medium (<8 cm) 3=Long (8-10 cm beyond ear tip) 4=Very Long (>10 cm)			2	

	7b. EAR (Husked Ear Data):	Standard Deviation	Sample Size	Standard Variety	Standard Deviation	Sample Size
16.7	cm Ear Length	01.15	03	16.0	02.65	03
40.0	mm Ear Diameter at mid-point	00.00	03	34.7	00.58	03
132.0	gm Ear Weight	07.21	03	83.7	13.58	03
15	Number of Kernel Rows	01.15	03	11.0	01.00	03
1	Kernel Rows: 1=Indistinct 2=Distinct			2		
2	Row Alignment: 1=Straight 2=Slightly Curved 3=Spiral			1		
08.3	cm Shank Length	01.15	03	09.0	01.73	03
2	Ear Taper: 1=Slight 2= Average 3=Extreme			1		

	8. KERNEL (Dried)	Standard Deviation	Sample Size	Standard Variety	Standard Deviation	Sample Size
10.7	mm Kernel Length	00.58	03	10.7	00.58	03
07.7	mm Kernel Width	00.58	03	09.0	00.00	03
04.7	mm Kernel Thickness	00.58	03	05.0	00.00	03
49.0	% Round Kernels (Shape Grade)	08.89	03	60.0	01.73	03
1	Aleurone Color Pattern: 1-Homozygous 2=Segregating			1		
07	Aluerone Color (Munsell code)	10YR714		07	10YR814	
07 ~~10~~	Hard Endosperm Color (Munsell code)	10YR612		07	10YR714	
03	Endosperm Type: 1=Sweet (Su1) 2=Extra Sweet (sh2) 3=Normal Starch 4=High Amylose Starch 5=Waxy Starch 6=High Protein 7=High Lysine 8=Super Sweet (se) 9=High Oil 10=Other_			3		
28.0	gm Weight per 100 Kernels (unsized sample)	01.73	03	32.00	01.00	03

	9. COB:	Standard Deviation	Sample Size	Standard Variety	Standard Deviation	Sample Size
24.3	mm Cob Diameter at mid-point	00.58	03	19.3	01.15	03
11	Cob Color (Munsel code) 2.5YR76			14	2.5YR56	

JMS 4/21/04

10. DISEASE RESISTANCE (Rate from 1 (most susceptible) to 9 (most resistant); leave blank if not tested; leave Raceor Strain Options blank if polygenic):

A. Leaf Blights, Wilts, and Local Infection Diseases

	Anthracnose Leaf Blight (Colletotrichum graminicola)	
6	Common Rust (Puccinia sorghi)	6
	Common Smut (Ustilago maydis)	
	Eyespot (Kabatiella zeae)	
	Goss's Wilt (Clavibacter michiganense spp. nebraskense)	
5	Gray Leaf Spot (Cercospora zeae-maydis)	5
	Helminthosporium Leaf Spot (Bipolaris zeicola) Race ——	
7	Northern Leaf Blight (Exserohilum turcicum) Race ——	5
5	Southern Leaf Blight (Bipolaris maydis) Race ——	6
	Southern Rust (Puccinia polysora)	
	Stewart's Wilt (Erwinia stewartii)	
	Other (Specify) ——	

B. Systemic Diseases

	Corn Lethal Necrosis (MCMV and MDMV)	
6	Head Smut (Sphacelotheca reiliana)	9
	Maize Chlorotic Dwarf Virus (MDV)	
	Maize Chlorotic Mottle Virus (MCMV)	
	Maize Dwarf Mosaic Virus (MDMV)	
	Sorghum Downy Mildew of Corn (Peronosclerospora sorghi)	
	Other (Specify) ——	

C. Stalk Rots

6	Anthracnose Stalk Rot (Colletotrichum graminicola)	3
	Diplodia Stalk Rot (Stenocarpella maydis)	
	Fusarium Stalk Rot (Fusarium moniliforme)	
	Gibberella Stalk Rot (Gibberella zeae)	
	Other (Specify) ——	

D. Ear and Kernel Rots

	Aspergillus Ear and Kernel Rot (Aspergillus flavus)	
7	Diplodia Ear Rot (Stenocarpella maydis)	6
5	Fusarium Ear and Kernel Rot (Fusarium moniliforme)	7
9	Gibberella Ear Rot (Gibberella zeae)	8
	Other (Specify) ——	

11. INSECT RESISTANCE (Rate from 1 (most susceptible) to 9 (most resistant); (leave blank if not tested) :

Banks grass Mite (Oligonychus pratensis)
Corn Worm (Helicoverpa zea)
  Leaf Feeding
  Silk Feeding
    mg larval wt.
Ear Damage
Corn Leaf Aphid (Rhopalosiphum maidis)
Corn Sap Beetle (Carpophilus dimidiatus)
European Corn Borer (Ostrinia nubilalis)
  1st Generation (Typically Whorl Leaf Feeding)
  2nd Generation (Typically Leaf Sheath-Collar Feeding)
  Stalk Tunneling
  cm tunneled/plant
Fall Armyworm (Spodoptera fruqiperda)
  Leaf Feeding
  Silk Feeding
  mg larval wt.
Maize Weevil (Sitophilus zeamaize
Northern Rootworm (Diabrotica barberi)
Southern Rootworm (Diabrotica undecimpunctata)
Southwestern Corn Borer (Diatreaea grandiosella
  Leaf Feeding
  Stalk Tunneling
  cm tunneled/plant
Two-spotted Spider Mite (Tetranychus urticae)
Western Rootworm (Diabrotica virgifrea virgifea)
Other (Specify) ——

12. AGRONOMIC TRAITS:

Application	Trait	Standard
4	Staygreen (at 65 days after anthesis) (Rate on a sale from 1=worst to excellent)	3
	% Dropped Ears (at 65 days after anthesis)	
	% Pre-anthesis Brittle Snapping	
	% Pre-anthesis Root Lodging	
90.0	Post-anthesis Root Lodging (at 65 days after anthesis)	13.3
7,032.3	Kg/ha Yield of Inbred Per Se (at 12-13% grainmoisture)	4,261.0

13. MOLECULAR MARKERS: (0=data unavailable; 1=data available but not supplied; 2=data supplied):

1 Isozymes   0 RFLP's   0 RAPD's

COMMENTS (eg. state how heat units were calculated, standard inbred seed source, and/or where data was collected. Continue in Exhibit D):

CLARIFICATION OF DATA IN EXHIBITS B AND C 20020018 7

Please note the data presented in Exhibit B and C, "Objective Description of Variety," are collected primarily at Johnston and Dallas Center, Iowa. The data in Tables 1A and 1B are from two sample t-tests using data collected in Johnston and Dallas Center, IA. These traits in exhibit B collectively show distinct differences between the two varieties.

JMS 10/22/03

The data collected in exhibit C was collected in 2001 for page 1 and 2. There were 3 different planting dates planted for these trials. There are environmental factors that differ from planting date to planting date. Environmental temperature and precipitation differences during the vegetative and grain fill periods can impact plant and grain traits, and are a source of variability. The environmental conditions described above could result in larger standard deviations. The variation associated with environment to environment is normally higher than the variation associated within locations. Also, the ear and sizing traits can vary depending on how well pollinated the ears are and how consistent the weather is during the grain fill period. I have enclosed a table that shows monthly temperature and precipitation in 2001.

20020187

Exhibit ~~D~~ C. Temperature and Precipitation differences from Ankeny, IA

TEMPERATURE

YEAR	MAY	JUN	JULY	AUG	AVERAGE
1994	59.8	70.7	71.9	69.0	67.9
1995	56.2	69.4	74.3	76.9	69.2
1996	56.2	69.3	71.3	70.5	66.8
1997	53.5	70.6	74.1	69.6	67.0
1998	64.7	66.6	74.8	73.5	69.9
1999	60.7	69.7	78.7	70.5	69.9
2000	63.5	68.9	73.2	74.2	70.0
2001	61.3	69.0	76.7	74.2	70.3
2002	57.7	73.5	77.9	71.7	70.2

RAINFALL

YEAR	MAY	JUN	JULY	AUG	Total
1994	3.67	5.75	1.71	4.18	15.31
1995	5.04	4.19	2.94	2.87	15.04
1996	8.47	4.35	2.51	2.14	17.47
1997	4.32	3.27	4.10	1.36	13.05
1998	6.46	11.07	5.70	4.96	28.19
1999	6.46	4.54	4.45	6.55	21.85
2000	5.40	5.80	3.16	1.78	16.14
2001	5.72	3.87	2.05	1.92	13.56
2002	2.91	2.78	5.34	4.00	15.03

U.S. DEPARTMENT OF AGRICULTURE
AGRICULTURAL MARKETING SERVICE

EXHIBIT E
STATEMENT OF THE BASIS OF OWNERSHIP

The following statements are made in accordance with the Privacy Act of 1974 (5 U. S. C. 552a) and the Paperwork Reduction Act (PRA) of 1995.

Application is required in order to determine if a plant variety protection certificate is to be issued (7 U.S.C. 2421). Information is held confidential until certificate is issued (7 U.S.C. 2426).

1. NAME OF APPLICANT(S)	2. TEMPORARY DESIGNATION OR EXPERIMENTAL NUMBER	3. VARIETY NAME
PIONEER HI-BRED INTERNATIONAL, INC.		**PH8CW**

4. ADDRESS *(Street and No., or R.F.D. No., City, State, and ZIP, and Country)*	5. TELEPHONE *(include area code)*	6. FAX *(include area code)*
7301 NW 62nd AVENUE P.O.BOX 85 JOHNSTON, IA 50131-0085	**515-270-4051**	**515-253-2125**
	7. PVPO NUMBER **200200187**	

8. Does the applicant own all rights to the variety? *Mark an "X" in appropriate block.* ***If no, please explain:*** ☒ YES ☐ NO

9. Is the applicant (individual or company) a U.S. national or U.S. based company? ☒ YES ☐ NO

If no, give name of country

10. Is the applicant the original owner? ☒ YES ☐ NO ***If no, please answer one of the following:***

a. If original rights to variety were owned by individual(s), is(are) the original owner(s) a U.S. national(s)?

☐ YES ☐ NO ***if no, give name of country***

b. If original rights to variety were owned by a company(ies), is(are) the original owner(s) a U.S. based company?

☒ YES ☐ NO ***If no, give name of country***

11. Additional explanation on ownership *(if needed, use reverse for extra space)*:

PH8CW is owned by Pioneer Hi-Bred International, Inc.

Pioneer Hi-Bred International, Inc. (PHI), Des Moines, Iowa, and/or its wholly owned subsidiary Pioneer Overseas Corporation (POC), Des Moines, Iowa, is the employer of the plant breeders involved in the selection and development of PH8CW. Pioneer Hi-Bred International and/or Pioneer Overseas Corporation has the sole rights and ownership of PH8CW pursuant to written contracts that assign all rights in the variety to PHI and/or POC at the time such variety was created. No rights to this variety are retained by any individuals.

JMC 10/22/03

PLEASE NOTE:

Plant variety protection can be afforded only to owners (not licensees) who meet one of the following criteria:

1. If the rights to the variety are owned by the original breeder, that person must be a U.S. national, national of a UPOV member country, or national of a country Which affords similar protection to nationals of the U.S. for the same genus and species.
2. If the rights to the variety are owned by the company which employed the original breeder(s), the company must be U.S. based, owned by nationals of a UPOV member country, or owned by national of a country which affords similar protection to nationals of the U.S. for the same genus and species.
3. If the applicant is an owner who is not the original owner, both the original owner and the applicant must meet one of the above criteria.

The original breeder/owner may be the individual or company who directed final breeding. See section 41(a)(2) of the Plant Variety Protection Act for definition.

STD-470-E (07-97) *(Destroy previous editions).*
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